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# DIRECT CRYSTALLIZATION OF LYSOZYME FROM EGG WHITE AND SOME CRYSTALLINE SALTS OF LYSOZYME

By GORDON ALDERTON AND H. L. FEVOLD

(From the Western Regional Research Laboratory,\* Albany, California)

(Received for publication, March 13, 1946)

A method of isolating and crystallizing lysozyme, the bacteriolytic substance in egg white, has been reported from this Laboratory (1). Crystallization was effected from acid solutions of purified material containing approximately 5 per cent salt, and it was also accomplished in the isoelectric region (pH 9.5 to 11.0) as a result of markedly lower solubility of the crystalline product in that region. Since that report was made, methods have been improved and lysozyme has been crystallized as the salt of several acids. In addition it has been crystallized directly from egg white by a method which markedly facilitates isolation in pure form.

## *Crystal Forms of Lysozyme*

The previous report (1) states that the crystal form of lysozyme varied with the pH of crystallization and the acid radical with which it was combined. Table I presents briefly the results obtained when electrodyalyzed, isoelectric lysozyme was crystallized after it had been dissolved to various pH values with hydrochloric acid in 5 per cent sodium chloride solution. Two forms of crystals were obtained. The form shown in Fig. 1 was obtained below pH 7.0; between pH 7.0 and 11 the type shown in Fig. 2 was obtained.<sup>1</sup> In appearance the crystals formed at any pH above 7.0 seem identical. They differ chemically, however, in that those formed in the isoelectric region are relatively insoluble in water, whereas those formed below pH 9.0 are increasingly soluble with decreasing pH. Apparently enough of the basic groups are combined with acid to make the product soluble, but the crystal form remains unchanged. Below pH 7.0, the form is markedly different and a form typical of lysozyme chloride is obtained, presumably because of combination of more and varied basic groups with acid.

Crystallization at the isoelectric point is readily accomplished by adjusting a 5 per cent solution of amorphous lysozyme chloride in 5 per cent sodium chloride solution to pH 10.5 with sodium hydroxide. No pre-

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

<sup>1</sup> In some of the preparations at acid pH (4.8 or above) some needle-like crystals formed after standing for some time.



cipitation of amorphous material takes place, but on standing crystalline material begins to separate and continues to do so until the lysozyme in solution is reduced to approximately 0.1 per cent. The presence of increasing amounts of sodium chloride up to 5 per cent increases the amount

TABLE I

*Effect of pH on Crystallization of Lysozyme*

Original protein concentration, 4 per cent; salt concentration, 5.0 per cent.

pH	Degree of crystallization*	Crystal form
2.0	No crystals or ppt.	
3.5	Copious crystallization	Fig. 1
4.8	Moderate " "	" 1
5.8	Very slight crystallization	" 1
7.2	" " "	" 2
8.2	Copious crystallization	" 2
9.2	" " "	" 2
10.0	" " "	" 2
11.3	" " "	" 2

\* Visual estimation after 48 hours of crystallization.

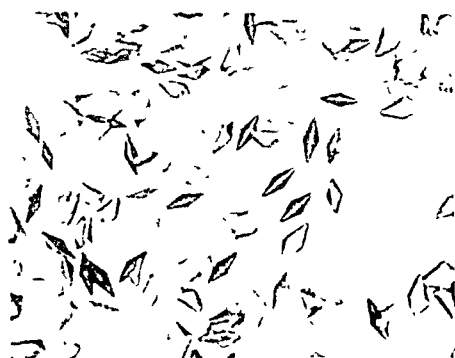


FIG. 1. Lysozyme hydrochloride; pH 4.5.  $\times 80$ .



FIG. 2. Isoelectric lysozyme.  $\times 80$

of amorphous material that can be dissolved in the isoelectric region, but less crystalline material may be dissolved in 5 per cent salt than in lower salt concentrations.

Lysozyme has also been crystallized as the iodide (Fig. 3) and the bromide (Fig. 4) by dissolving 200 mg. samples of electrodyalyzed isoelectric lysozyme in 10 ml. of solution of the appropriate acid at acid pH (bromide pH 6.0, iodide pH 4.0) and adding 5 per cent of the corresponding sodium

or potassium halide. On standing at room temperatures, crystals were deposited from both solutions. These crystals were needle-like, as shown, and thus were different in form from those obtained when hydrochloric acid was used at the same pH.<sup>2</sup>

Crystallization of lysozyme from a 1 per cent protein solution in nitric acid at pH 4.0 containing 5 per cent potassium nitrate resulted in the crystal form shown in Fig. 5. These crystals are different in form from any of the others.<sup>3</sup>

The most rapid crystallization of lysozyme was effected from bicarbonate solutions. Isoelectric lysozyme readily dissolves in carbonic acid and, when sodium bicarbonate (5 per cent) was added, crystallization began within a few minutes. The crystals were well defined, small needles (Fig. 6).

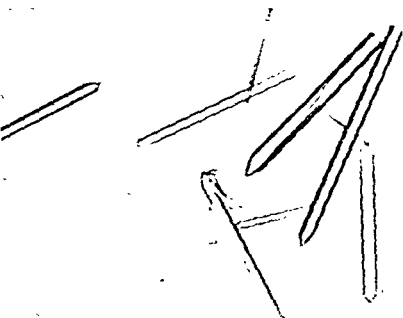


Fig. 3. Lysozyme hydroiodide.  $\times 80$

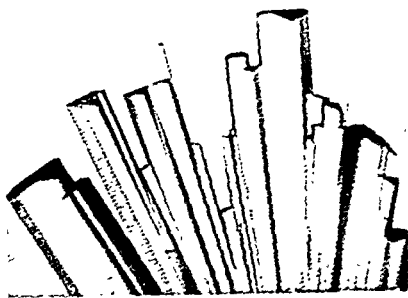


Fig. 4. Lysozyme hydrobromide;  
pH 6.  $\times 80$ .

Attempts have been made to crystallize other salts of lysozyme, such as the acetate, sulfate, and tartrate, without success. The first crystalline lysozyme preparation was obtained from an acetate buffer on addition of ammonium sulfate but we have never been successful in obtaining crys-

<sup>2</sup> Determination of the combined chloride in the lysozyme hydrochloride crystals prepared at pH 4.3 and 8.2 gave values of 3.24 and 1.99 per cent chlorine, respectively. With 17,500 as the molecular weight for lysozyme, sixteen and ten basic groups are therefore combined with hydrochloric acid at pH 4.3 and 8.2, respectively. Similar analyses of the hydrobromide crystals at pH 4.0 gave 7.6 per cent bromine, or sixteen to seventeen combined groups. These values were arrived at by determining total halogen and sodium in the crystalline preparations and then calculating the combined halogen by subtracting the amount of halogen equivalent to the cation.

<sup>3</sup> Crystallographic characterization of the crystal forms will be reported by F. T. Jones of this Laboratory.

talline material from a system which contained only the sulfate or the acetate anion.

### *Direct Crystallization of Lysozyme from Egg White*

The fact that 5 per cent solutions of lysozyme in 5 per cent sodium chloride at or near the isoelectric point readily yielded crystalline material, and that the crystalline material is only slightly soluble under these conditions, suggested the possibility that crystallization could be induced from egg white directly.

It was found in the initial experiments that egg white, freed of chalazae and homogenized, with adjustment to pH 10.8 and addition of 5 per cent sodium chloride, yielded no precipitate and no crystalline material when allowed to stand for several days. However, when a suspension of iso-

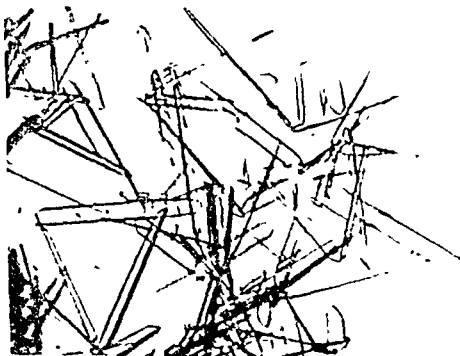


FIG. 5. Lysozyme nitrate.  $\times 80$



FIG. 6. Lysozyme carbonate.  $\times 80$

electric crystalline lysozyme was added to the egg white solution under the same conditions, crystallization was induced and 50 per cent of the lysozyme of the egg white was obtained as fine needle-like crystals.

In an attempt to improve the yield, a series of experiments was carried out to determine the effect on crystallization of pH in the isoelectric region, salt concentration, and temperature. The alkalinity was adjusted as desired by addition of 1  $N$  potassium hydroxide. The results (Table II) show that the yield of crystalline material was markedly affected by the temperature of crystallization. Very low yields were obtained at  $-8^{\circ}$  and  $23^{\circ}$ , as compared with  $4^{\circ}$ . Also, a pH of 9 to 9.5 resulted in more rapid crystallization than when higher alkalinities were used.

Yields of 60 to 80 per cent of the total lysozyme contained in egg white were regularly obtained after egg white had been allowed to stand for 3 to 4 days at  $4^{\circ}$ . The egg white, after adjustment to pH 9.5 and addition of

5 per cent sodium chloride, must be seeded with small amounts of crystalline lysozyme. Occasionally crystallization will set in without seeding but not in most instances. The crystalline product thus obtained is redissolved in acetic acid (pH 4 to 6) and any insoluble material is removed by centrifugation and discarded. The soluble material is then recrystallized after addition of 5 per cent sodium chloride and adjustment to pH 9.5 to 11.0. This recrystallization can also be quickly accomplished by making the acid solution of the crystals 5 per cent with respect to sodium bicarbonate (pH 8.0 to 8.5).

TABLE II

*Effect of Salt Concentration, pH, Temperature, and Time on Crystallization of Lysozyme from Egg White*

Effect of salt concentration (pH 10.5, 4°)			Effect of pH (salt concentration 5 per cent, 4°)			Effect of temperature (pH 10.5, salt concentration 5 per cent)		
Salt	Yield*		pH	Yield*		Temperature	Yield*	
	20 hrs.	72 hrs.		20 hrs.	72 hrs.		20 hrs.	72 hrs.
per cent	per cent of total	per cent of total		per cent of total	per cent of total	°C.	per cent of total	per cent of total
0	0	5	9.0	72	86	-8	5	23
1	2	7	9.5	76	88	4	12	75
2	5	11	10.0	62	84	23	5	12
4	16	62	10.5	18	75			
5	18	75	11.0	10	64			
6	10	74						
8	6	72						

\* Determined by the assay method of Boasson (2).

## SUMMARY

Lysozyme has been crystallized over the pH range of 3 to 11 from 5 per cent sodium chloride solutions. Two distinct crystal forms were obtained, one below pH 7.0 and the other at basic reactions. The chloride, bromide, iodide, nitrate, and carbonate of lysozyme have been prepared in crystalline form.

A method of crystallizing lysozyme from egg white directly has been described. Yields of 60 to 80 per cent were obtained:

We are indebted to F. T. Jones for photomicrographs of the crystalline material, and to L. M. White and C. M. Johnson for the halogen and sodium analyses.

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2. Boasson, E. H., *J. Immunol.*, **34**, 281 (1938).



# BEHAVIOR OF DEXTRINS TOWARDS TAKA-DIASTASE

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(Received for publication, February 21, 1946)

That taka-dia-*stase*, when allowed to react under the appropriate conditions, will hydrolyze starch and maltose completely or almost completely to glucose has been known for some time (2, 9). It might, therefore, be expected that dextrans, which are intermediates in the hydrolytic breakdown of starch to maltose, should be similarly susceptible to taka-dia-*stase*. Results contrary to this expectation were obtained in a series of experiments carried out by the writer.

Solutions of starch, dextrans, and maltose were prepared, containing 125 mg. of the carbohydrate per 100 ml. of solution. The starch and dextrin solutions were boiled for 3 minutes before being cooled and made to volume. For the digestion 20 ml. aliquots of the solutions (containing 25 mg. of carbohydrate) were mixed in Erlenmeyer flasks with 20 ml. of thymol-saturated acetic acid-sodium acetate buffer solution, pH 4.45, and 10 ml. of dialyzed taka-dia-*stase* solution, corresponding to 50 mg. of original taka-dia-*stase* (Parke, Davis and Company, Detroit, Michigan). The flasks were tightly stoppered and incubated for 44 hours at 37-38°. Blank digests, in which the carbohydrate solution was replaced by an equal volume of distilled water, were included in all series. At the end of the digestion period the reducing power of the digests was determined by a previously described semimicromethod (8). A 20 ml. aliquot of each digest was then hydrolyzed with 1.6 ml. of 25 per cent hydrochloric acid for 1 hour in an autoclave at 15 pounds pressure. This treatment is known to result in the complete breakdown of starch and dextrans to glucose (3). The hydrolysates were cooled down, transferred to 50 ml. standard flasks, neutralized, and made to volume. The reducing power was then again determined. By expressing the reducing power after taka-dia-*stase* digestion as a percentage of the reducing power after acid hydrolysis (with the necessary corrections for blank values and dilutions) a measure of the degree of completeness of the taka-dia-*stase* digestion was obtained. The results are given in Table I.

As will be seen, all the dextrans proved more or less resistant to taka-dia-*stase* under conditions under which starch and maltose were almost completely hydrolyzed. The degree of resistance increased with the darkness in color of the dextrans, and remarkably similar results were obtained

with dextrans of the same color but of different origin. Experiments were also carried out in which mixtures of dextrin and maltose, and of dextrin, maltose, and glucose were subjected to taka-diaztase digestion. While it is not necessary to give the results of these experiments in detail, it may be mentioned that the presence of maltose and glucose did not affect the degree of digestibility of the dextrin.

Since it was not certain whether equilibrium in the dextrin digests had been established after 2 days, a further experiment was carried out with two of the dextrans in which the digestion was continued for 14 days. The technique was the same as previously employed but samples for sugar determinations were taken from the digests after 2, 4, 7, 11, and 14 days.

TABLE I  
*Effect of Taka-diaztase upon Starch, Dextrans, and Maltose*

Carbohydrate	Glucose found per digest		Per cent digested by taka diaztase
	Before acid hydrolysis	After acid hydrolysis	
	mg	mg	
Rice starch*	25.4	26.6	95.5
Maize starch, technical†	23.0	23.4	98.3
White dextrin, British Drug Houses, technical	21.1	24.3	86.8
"African Maid"†	20.9	24.7	84.6
Yellow dextrin, Merck's*	13.8	26.2	52.7
"African Maid"†	11.3	24.5	46.1
Dark yellow dextrin, "African Maid"†.	6.3	24.7	25.5
Maltose, Schering-Kahlbaum*	24.5	25.9	94.5

\* Dried *in vacuo* over phosphoric oxide.

† Manufactured and kindly supplied by the African Products Manufacturing Company, Ltd., Germiston, near Johannesburg, South Africa. Dextrans produced from maize starch by hydrolysis with hydrochloric acid.

Table II gives the glucose values, expressed as percentages of the previously determined glucose value of the acid hydrolysate.

The results indicated that, while equilibrium was evidently not reached after 2 days, further breakdown took place at a very slow rate. From the 2nd to the 14th day the reducing power of the two digests increased by only 4.1 and 2.5 per cent respectively. The reaction was obviously still proceeding when the experiment was discontinued.

Somewhat similar results were obtained by some previous workers. Sahyun and Alsberg (5) found that glycogen was only partly broken down by taka-diaztase, approximately 52 per cent of the glycogen ultimately appearing as reducing sugars. According to Ahlberg and Myrback (1)

the yield of limit dextrans produced by the action of taka-dia-*stase* upon corn-starch was nearly 20 per cent of the starch. Though these dextrans could still be further hydrolyzed by taka-dia-*stase*, the rate of the reaction was far less than 1/1000 of the digestion rate of starch. Certain dextrin fractions obtained from starch by acid hydrolysis were likewise highly resistant to taka-dia-*stase* (4). These authors, however, did not acidify the digestion mixtures, and used very small amounts of enzyme in relation to the amount of substrate; so that their results are not directly comparable with those of the present writer.

It remained to be shown whether, under the conditions described here, dextrans derived from starch by enzymatic activity behaved in the same way towards taka-dia-*stase* as commercial dextrans produced from starch by acid hydrolysis. Saliva is known to hydrolyze starch to a mixture of dextrans, maltose, and glucose; the latter is usually only formed in small amounts, except if saliva is present in considerable excess or after prolonged periods of contact (6, 7).

TABLE II  
*Prolonged Action of Taka-dia-*stase* upon Dextrans*

The values represent per cent dextrin digested.

	2 days	4 days	7 days	11 days	14 days
Yellow dextrin*.....	51.4	52.6	53.6	54.3	55.5
Dark yellow dextrin*.....	28.8	29.2	30.1	30.3	31.3

\* African Products Manufacturing Company.

Three 50 ml. aliquots of a solution of previously gelatinized rice starch, each containing 200 mg. of starch, were digested with 1 ml. of fresh saliva, under the following conditions: Digest 1, for 4 hours at room temperature; Digest 2, for 12 hours at room temperature; Digest 3, for 22 hours at 38°.

At the end of the digestion period the digests were boiled to destroy the enzyme, and made to 100 ml. volume. Qualitative tests with iodine solution indicated that Digest 1 still contained small amounts of starch, and Digest 2 traces of starch, whereas Digest 3 was starch-free. A portion of each digest was then treated with taka-dia-*stase*, after which an aliquot of each taka-dia-*stase* digest was subjected to acid hydrolysis (as in the first series of experiments). Reducing sugar determinations were made at all stages; the results are shown in Table III.

In the calculation of the maltose and dextrin values of the starch-saliva digests glucose was disregarded. Maltose was obtained by multiplying the apparent glucose value by the factor 1.96, previously determined by the writer (8). Dextrin was calculated as the difference (glucose after acid



hydrolysis minus maltose)  $\times 0.9$ . According to these calculations from one-fifth to one-third of the carbohydrate in the starch-saliva digests was in the form of dextrans. Since some glucose was probably present in the digests, the values of maltose are likely to be overestimated and those of dextrin underestimated. In contrast to the commercial dextrans, which proved more or less resistant to taka-diastrase, the dextrans produced by the action of ptyalin on starch were highly susceptible to taka-diastrase, which hydrolyzed them almost completely.

TABLE III

*Effect of Taka-diastrase on Maltose-Dextrin Mixtures Obtained by Action of Saliva upon Rice Starch*

The values represent mg. per 100 ml. of saliva digest.

Digest No.	Maltose after saliva digestion	Dextrin after saliva digestion	Glucose after taka-diastrase digestion	Glucose after acid hydrolysis	Per cent digested by taka-diastrase
1	143.1	65.6*	200.0	216.0	92.6
2	149.2	60.2*	200.8	216.0	93.0
3	168.2	39.4	200.8	212.0	94.8

\* Including small amounts of starch.

## SUMMARY

Commercial dextrans were found to be resistant to taka-diastrase, being only partly hydrolyzed by this enzyme under conditions which resulted in almost complete hydrolysis of starch and maltose.

The degree of resistance increased with darkness in color of the dextrans, and was similar for dextrans of the same color but of different origin.

Maltose and dextrin, formed by the action of saliva upon rice starch, were almost completely hydrolyzed by taka-diastrase.

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# STRUCTURAL ANALOGUES ANTAGONISTIC TO THYROXINE

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Since many compounds related structurally in specific ways to metabolically important substances have been found to produce signs of deficiency of the analogous metabolite, Woolley (1) proposed that new series of pharmacological agents might be produced by altering the structures of vitamins or hormones in definite ways. It was considered possible that such inhibitory structural analogues could be formed against certain hormones which, as a result of overproduction, or diminished rate of destruction in the body, are the causative agents of disease. The excess hormone might be counterbalanced by administration of the structurally related antagonist (compare (1)). As a first step towards an experimental test of this postulate it would be necessary to develop a compound which would antagonize the action of a hormone in a suitable biological test. Up to the present, inhibitory structural analogues of hormones have not been produced in any number.

Several new ethers of N-acetyldiiodotyrosine have been synthesized, and some have been found to counteract the pharmacological effects of thyroxine on tadpoles. The relationship of these ethers to the hormone follows from the fact that thyroxine is the hydroxydiiodophenyl ether of diiodotyrosine. The formation of the first members of the present series of agents came as a result of attempts to synthesize analogues of thyroxine with 1 or more C atoms interposed between the two benzene rings but otherwise identical with the hormone.

The synthesis of the desired *p*-hydroxydiiodobenzyl ether failed because all attempts to reduce the *p*-nitrobenzyl ether of N-acetyldiiodotyrosine to the amine (which could be deaminated and iodinated) led to reduction of the benzyl ether to N-acetyldiiodotyrosine. When the corresponding *p*-nitrophenylethyl ether was reduced, the amine was formed, but difficulties in the deamination made the synthesis of the phenylethyl analogue of thyroxine, *i.e.* *p*-hydroxydiiodophenylethyl ether of diiodotyrosine, highly impractical.

The nitrobenzyl ether was then tested for its antagonistic action to thyroxine, and, since it exhibited considerable potency in this respect, a number of other ethers of N-acetyldiiodotyrosine were prepared and tested.

\* With the technical assistance of M. L. Collyer, R. Brown, and A. Holloway. The elementary analyses were performed by Dr. E. Elek.

The presence of the *p*-nitro group (which was presumably reduced to an  $\text{NH}_2$  by the animal) was not necessary for activity, because the benzyl ether of N-acetyldiiodotyrosine was almost as potent as the *p*-nitrobenzyl ether. Indeed, an aromatic ring system was not essential, because the butyl ether displayed activity. It was necessary, however, to have some sort of ether, for N-acetyldiiodotyrosine itself was without detectable effect. Too close structural relationship to thyroxine did not seem desirable, since the methyl ether of N-acetylthyroxine was inactive as an antagonist.

In addition to their antithyroxine action, sufficiently large amounts of the ethers showed a toxic effect when tested on tadpoles in the absence of added thyroxine. Individual members of the series differed considerably in this respect. The *p*-nitrobenzyl or butyl ethers were of very low toxicity, while the *p*-nitrophenylethyl ether was the most poisonous.

Some, but not all, of the ethers, when tested in the absence of added thyroxine, showed slight hormone action. The *p*-nitrobenzyl ether was most active in this regard, while the butyl or benzyl ethers were without effect. While this work was being completed, Shive and Snell (2) reported a similar finding for the pantothenic acid activity of an inhibitory analogue of that vitamin. Such dual rôles of analogues must be considered in the formation of any hypothesis for the mode of action of these agents.

Although an antagonism between certain ethers of N-acetyldiiodotyrosine and thyroxine could be demonstrated, the ability of these agents to correct the manifestations of endogenously produced hormone (*i.e.*, hyperthyroidism) cannot be stated until further work is done. The *p*-nitrobenzyl ether was well tolerated when given orally to mice, but levels sufficient for therapeutic purposes may not have been maintained in the tissues.

#### EXPERIMENTAL

*p*-Nitrobenzyl Ether of N-Acetyl-3,5-diiodo-*l*-tyrosine—24 gm. of N-acetyl-3,5-diiodo-*l*-tyrosine<sup>1</sup> were dissolved in 110 cc. of 1 N NaOH. The solution was heated in a boiling water bath, stirred vigorously, and treated with 11 gm. (1 equivalent) of *p*-nitrobenzyl bromide, added in small portions during a period of 5 minutes. Heating and stirring were continued for 15 minutes, and the clear solution was then cooled to 0° for several hours. It was essential that no excess of the bromide was used, for, when this was done, a product low in iodine and high in nitrogen was obtained. As the

<sup>1</sup> The N-acetyl-3,5-diiodo-*l*-tyrosine was prepared according to the method of Myers (3). However, the melting point was 124° instead of the 198–200° recorded in the literature. The preparations were analytically pure (calculated, N 2.96; found, N 3.00), and were converted in good yield to the *p*-nitrobenzyl ether and back to the original without alteration of the melting point. All melting points recorded in this paper were uncorrected.

reaction mixture cooled, the sodium salt of the desired ether precipitated. This salt was separated in a centrifuge, washed with a small amount of cold water, and recrystallized from the minimal amount of 1  $\times$  NaOH. It was then dissolved in 750 cc. of water, and the solution was extracted once with ether, freed of ether, and then acidified with HCl. The *p*-nitrobenzyl ether of *N*-acetyldiiodotyrosine which precipitated was filtered off, washed, and dried. Yield 25 gm.; m.p. 82–87°. It began to soften at 82°, and sintered as the temperature was raised, until complete melting occurred at 87°. The rather wide melting range was found for most of the ethers prepared in this study. The *p*-nitrobenzyl ether could be recrystallized from dilute alcohol, but from this solvent it usually precipitated as an oil which crystallized only on long standing. Purification by this means did not alter the melting point.

$C_{11}H_{11}O_2N_2I_2$ . Calculated, N 4.6, I 41.6; found, N 4.7, I 40.8

Attempts to reduce the nitrobenzyl ether to the corresponding amine always led to the formation of *N*-acetyldiiodotyrosine, m.p. 124°, in good yield, whether the reducing agent was  $FeSO_4$  and  $NH_4OH$ , or alkaline hydrosulfite, or Sn and HCl.

*p*-Nitrophenylethyl Bromide—To 100 cc. of fuming nitric acid cooled to –15° and stirred rapidly, 50 gm. of phenylethyl bromide were added dropwise. The reaction mixture was poured on 200 gm. of cracked ice, and the precipitate which formed was collected and recrystallized from alcohol. Yield 43 gm.; m.p. 64°.

$C_8H_9O_2NBr$ . Calculated, Br 34.8; found, Br 34.8

To demonstrate that the nitro group had been introduced into the para position, a small amount of the compound was oxidized with alkaline permanganate to *p*-nitrobenzoic acid, m.p. 233°, the same as that of an authentic sample.

*p*-Nitrophenylethyl Ether of *N*-Acetyl-3,5-diiodo-*L*-tyrosine—9.6 gm. of *N*-acetyl-3,5-diiodo-*L*-tyrosine were dissolved in 64 cc. of methanolic 1  $\times$  NaOH and treated with 9.2 gm. of *p*-nitrophenylethyl bromide. It was necessary to use an excess of the bromide, and to carry out the reaction in alcohol, because the yield was small when the conditions described above for the preparation of the nitrobenzyl ether were employed. The solution was refluxed for 2 hours, concentrated under reduced pressure to dryness, and the residue was warmed with 20 cc. of water and cooled to 0° for several hours. The sparingly soluble sodium salt of the nitrophenylethyl ether was separated and dissolved in water. This aqueous solution was extracted with ether to remove *p*-nitrophenylethyl alcohol, and, after the dissolved ether had been distilled from the aqueous phase under reduced pressure, the *p*-nitrophenylethyl ether was precipitated by acidification

with HCl. It was then recrystallized from dilute alcohol. Yield 3.5 gm.; m.p. 162°.

$C_{19}H_{18}O_6N_2I_2$ . Calculated, N 4.5, I 40.7; found, N 4.6, I 39.6

3.5 gm. of the ether were reduced by dissolving in 100 cc. of dilute  $NH_4OH$  at about 70° and slowly adding 11 gm. of  $FeSO_4 \cdot 7H_2O$  dissolved in water. The mixture was centrifuged and the resulting solution was concentrated under reduced pressure and adjusted to pH 4.5. Part of the amine precipitated as the ammonia was removed by distillation, and the remainder when the pH was brought to the isoelectric point. It formed a sparingly soluble hydrochloride with aqueous HCl. When attempts were made to deaminate this amine to the corresponding phenol according to standard methods, much darkening occurred, and only a small yield of what appeared to be the phenol was isolated from the reaction mixture. This was iodinated and the products separated according to the method of Harington and Barger (4), but the yield of the phenylethyl analogue of thyroxine so produced was only 121 mg., and did not allow adequate purification and study.

*Benzyl Ether of N-Acetyl-3,5-diiodo-L-tyrosine*—A solution of 4.8 gm. of N-acetyl-3,5-diiodo-L-tyrosine in 24 cc. of 1 N NaOH was stirred vigorously, heated in a boiling water bath, and treated with 1.3 gm. of benzyl chloride dropwise. On cooling the reaction mixture, the sodium salt of the benzyl ether, in contrast to the behavior of other ethers examined in this work, did not precipitate; so that it was necessary to acidify the reaction mixture, and to reprecipitate the product which separated from 35 per cent alcohol. This operation had to be repeated several times in order to remove completely the unchanged N-acetyldiiodotyrosine. The latter could be detected in this or other ethers herein described by the red color it gives with nitrous acid and ammonia (3). The benzyl ether was finally recrystallized from methanol. Yield 3.4 gm.; m.p. 84–90°.

$C_{18}H_{17}O_4NI_2$ . Calculated, N 2.5; found, N 2.7

*Butyl Ether of N-Acetyl-3,5-diiodo-L-tyrosine*—This compound was obtained from *n*-butyl bromide and N-acetyl-3,5-diiodo-L-tyrosine in a manner similar to that described for the *p*-nitrophenylethyl ether. Yield from 9.5 gm. of the tyrosine derivative 6.1 gm.; m.p. 103°.

$C_{18}H_{19}O_4NI_2$ . Calculated, N 2.7; found, N 2.7

*O-Methyl-N-acetyl-DL-thyroxine* was prepared according to the directions of Myers (3).

*Assay with Tadpoles for Detection of Antagonism to Thyroxine*—The assay was based on the fact that thyroxine is a lethal agent to tadpoles. As is well known, minute quantities of this hormone stimulate markedly the

rate of metamorphosis of these animals. Larger amounts cause rapid metamorphosis which is abruptly terminated by death. For the purposes at hand, protection against death proved to be a better criterion of anti-thyroxine activity than measurements of the rate of change into frogs. Furthermore, the aim of the work was to find agents which would protect against the toxic effects of excess thyroxine.

Groups of about twelve tadpoles, each animal 3 to 4 cm. in total length and in the stage of development just prior to the appearance of hind leg buds, were immersed in 500 cc. portions of a 1:20 dilution of frog-Ringer's solution<sup>2</sup> in distilled water. In each series of tests graded doses of thyroxine, of the compounds to be tested, and of a mixture of a fixed amount of thyroxine and graded doses of the substances to be tested were added. All compounds had been dissolved in an equivalent of NaOH, and the solutions brought to pH 7 with HCl. The tadpoles were admitted and after 24 hours were strained off, washed with water, and returned to 500 cc. of diluted Ringer's solution and fed a small piece of hard boiled egg white. Solution and food were changed daily. The animals were observed for signs of metamorphosis over a period of 2 weeks. During the test period, the untreated control groups which were always included in a series grew slightly (about 4 mm. average increase) but did not otherwise change noticeably.

The *p*-nitrobenzyl ether was tested for its effect on the quantitative assay for thyroxine proposed by Gaddum (5). This test depended on the shrinkage in total length, which followed the submergence of tadpoles in very dilute solutions of the hormone. The ability of the nitro ether to overcome this effect was taken as another measure of antagonistic action.

*Antagonism of Certain Thyroxine Analogues to Hormone As Measured by Tadpole Test*—When the *p*-nitrobenzyl ether of *N*-acetyl-3,5-diiodo-*l*-tyrosine was mixed with one or more lethal doses of thyroxine, the toxic action of the hormone was diminished or almost completely abolished (see Table I). Furthermore, the data show that the corresponding benzyl ether was slightly less, and the *p*-nitrophenylethyl ether slightly more active. The butyl ether was less active than the benzyl compounds. *N*-Acetyl-3,5-diiodo-*l*-tyrosine was ineffective, as was also *O*-methyl-*N*-acetyl-thyroxine.

*Thyroxine Action of Analogues*—The data in Table I also show that the *p*-nitrobenzyl ether when tested in the absence of the hormone had thyroxine activity. Measured by the quantitative technique of Gaddum (5), this ether had about 0.05 per cent of the activity of *dl*-thyroxine. The *p*-nitrophenylethyl ether and the benzyl ether had a barely perceptible thyroxine effect, but the butyl ether was inert in this respect. Because of this thyroxine activity it was not possible to achieve complete protection

<sup>2</sup> Glucose was omitted from this solution.

TABLE I

*Effects of Analogues of Thyroxine, with and without Hormone, on Rate of Survival and Metamorphosis of Tadpoles*

Compound		dl-Thyroxine	No. of animals	No. of deaths	Remarks on metamorphosis
	mg. per cc.	$\gamma$ per cc.			
None.....		0	74	3	No change
" .....		0.5	31	12	All had 4 legs
" .....		1.0	24	16	" " 4 "
" .....		1.2	19	16	" " 4 "
" .....		2.0	31	29	" " 4 "
" .....		3.0	22	22	" " 4 "
<i>p</i> -Nitrobenzyl ether*.....	0.50	0	19	0	5 had hind leg buds or legs
" " .....	0.75	2.0	12	1	Hind legs only
" " .....	0.50	2.0	24	7	All 4 legs
" " .....	0.37	1.0	23	8	" 4 "
" " .....	0.25	2.0	7	6	" 4 "
" " .....	0.25	1.0	11	6	" 4 "
" " .....	0.50	4.0	7	4	" 4 "
" " .....	0.50	0.5	12	0	Hind leg buds only
" " .....	0.50	10.0	12	11	All 4 legs, but survival 1 day longer than controls which received 10 $\gamma$ per cc. thyroxine alone
<i>p</i> -Nitrophenylethyl ether.....	0.50	0	11	4	3 had hind leg buds
" " .....	0.50	2.0	10	1	Hind legs only
" " .....	0.30	1.0	12	1	" " "
" " .....	0.25	1.0	12	4	" " "
Benzyl ether.....	0.75	0	7	2	" leg buds barely visible
" " .....	0.75	1.2	12	3	Hind legs only
" " .....	0.50	1.2	7	4	All 4 legs
" " .....	0.25	1.2	7	2	" 4 "
<i>n</i> -Butyl " .....	0.75	0	16	3	No change, deaths were from inter-current causes
" " .....	1.00	1.2	7	0	All 4 legs
" " .....	0.50	1.2	7	6	" 4 "
N-Acetyldiiodo- <i>l</i> -tyrosine.....	1.00	1.2	20	16	" 4 "
O-Methyl-N-acetyl- <i>dl</i> -thyroxine.....	0.10	1.2	12	12	" 4 "
" .....	0.50	1.2	12	12	All dead within 2 days

\* All ethers were of N-acetyl-3,5-diiodo-*l*-tyrosine.

of the tadpoles against accelerated metamorphosis with the nitro ethers because the amounts of these necessary for total counteraction of the test dose of thyroxine were enough to exert a small hormone effect by themselves.

*Poisonous Properties of Analogues*—Some of the thyroxine analogues showed a toxic action on tadpoles when tested in the absence of thyroxine. This manifested itself by death of the animals within 24 hours of their immersion in solutions of the agents. This was in contrast to any lethal effects attributable to thyroxine-like toxicity, in which characteristically death occurred several days after immersion, and after accelerated metamorphosis had run its course. The *p*-nitrophenylethyl ether was the strongest in this respect.

*Thyroxine Activity of p-Nitrobenzyl Ether in Mice*—The slight thyroxine activity of the *p*-nitrobenzyl ether was also found when it was assayed in mice for its ability to protect these animals against the toxic action of methyl cyanide (6). When fed at 20 mg. per gm. of diet, it protected 50 per cent of the mice from 1.5 lethal doses of methyl cyanide, an effect equal to that shown by about 1  $\gamma$  of *dl*-thyroxine per gm. of ration. The inclusion of 2 per cent of the nitrobenzyl ether in the diet for a period of 10 days did not seem to affect the mice adversely.

*Influence of p-Nitrobenzyl Ether on Quantitative Assay for Thyroxine with Tadpoles*—When tested according to the method of Gaddum (5), the activity of thyroxine in decreasing the length of tadpoles was reduced to 70 per cent of the original value by the inclusion of 0.5 mg. per cc. of the nitrobenzyl ether.

#### SUMMARY

Several ethers of *N*-acetyldiiodotyrosine were prepared for the first time and shown to protect tadpoles against the lethal action of thyroxine. They also antagonized the effect of the hormone in causing increased rate of metamorphosis of these animals. The *p*-nitrophenylethyl ether was the most active, followed closely by the *p*-nitrobenzyl ether. The benzyl and butyl ethers were less effective. The nitro ethers had a weak thyroxine activity (in either tadpoles or mice) as well as an antithyroxine property. *N*-Acetyldiiodotyrosine and *O*-methyl-*N*-acetylthyroxine were ineffective as antagonists of thyroxine.

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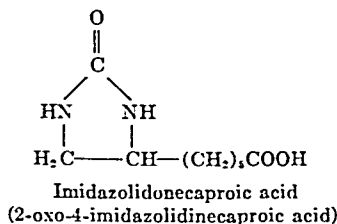
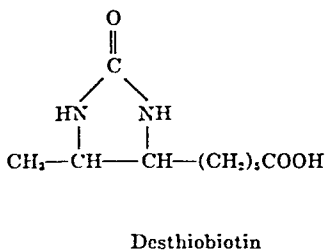
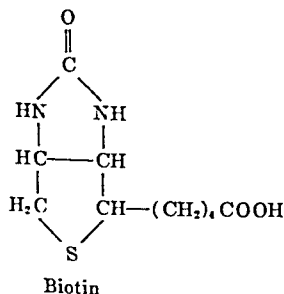
# SYNTHESIS OF IMIDAZOLIDONE ALIPHATIC ACIDS\*

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After we had demonstrated the presence of a cyclic urea ring in biotin (1, 2), the synthesis of cyclic urea compounds containing aliphatic carboxylic acid side chains was undertaken. It was hoped that these compounds might possess either biotin-like activity or antibiotin activity. We were also interested in finding out whether such cyclic urea compounds could combine with avidin.

This work received further stimulus when it was found that desthiobiotin (3) was as active as biotin for the stimulation of the growth of yeasts (4, 5). The possibility that the cyclic urea compounds with the carboxylic acid side chains might be antibiotics was strengthened when desthiobiotin was found to be an antibiotic for *Lactobacillus casei* (6) and several other



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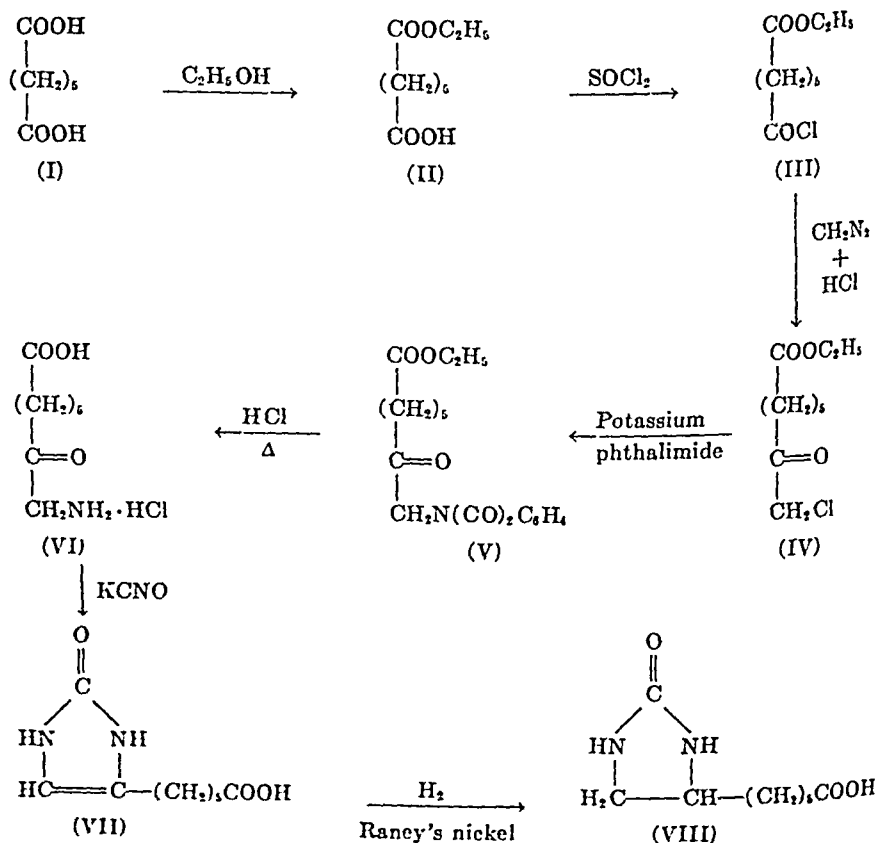
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microorganisms (7). When it was shown that yeast could synthesize biotin from desthiobiotin (6), the question arose as to whether the yeast could also introduce a methyl group into suitable precursors and thus make biotin from compounds even simpler than desthiobiotin.

In an attempt to answer some of these questions, four imidazolidone aliphatic acids have been prepared; namely, imidazolidonevaleric, imidazolidonecaproic, imidazolidoneheptanoic, and imidazolidoneoctanoic acids. The relationship between the structure of biotin, desthiobiotin, and imidazolidonecaproic acid is illustrated by the accompanying structures.

The microbiological activity of some of these imidazolidone aliphatic acids and a brief description of their synthesis have already been reported in a preliminary note (8). In the present paper we wish to present the details of the synthesis of these compounds. Data on their microbiological activity will appear in a subsequent communication.

The steps in the synthesis of imidazolidonecaproic acid (VIII) from pimelic acid (I) are shown in the accompanying reactions.



The imidazolidonevaleric acid was prepared from adipic acid in a similar manner. Likewise, imidazolidoneheptanoic and imidazolidoneoctanoic acids were prepared from suberic and azelaic acids, respectively.

## EXPERIMENTAL

### *Preparation of Imidazolidone Aliphatic Acids*

The monoethyl esters of the dicarboxylic acids were prepared according to the method described for the preparation of ethyl hydrogen sebacate (9). The half ester acid chlorides were obtained by treatment of the monoethyl esters with purified thionyl chloride, according to the usual procedure for this reaction.

*Ethyl 8-Phthalimido-7-oxooctanoate (V)*—For the preparation of the chloromethyl ketone used for the synthesis of (V) the half ester acid chloride was treated with diazomethane (10, 11) by the method described below. Diazomethane was prepared from nitrosomethylurea, according to the standard procedure (9 (p. 165)).

An ether solution of 0.13 mole of diazomethane was distilled over KOH pellets into a solution of 18.4 gm. (0.089 M) of ethyl  $\epsilon$ -chloroformyl caproate (III) in 200 cc. of absolute diethyl ether. The reaction flask, equipped with a stirrer and condenser, was cooled in an ice bath while the diazomethane was added. The reaction mixture was allowed to stand for about 20 hours, after which period dry HCl was passed into the solution with stirring. After no more nitrogen was evolved, the ether was removed *in vacuo*, a few cc. of absolute ethanol were added, and the solution was distilled *in vacuo*. The yield of crude ethyl 8-chloro-7-oxooctanoate (IV) was 17.7 gm. The crude chloromethyl ketone was converted directly to the phthalimido ester without further purification.

To 40 cc. of dry xylene were added 10 gm. of potassium phthalimide and 7.5 gm. of ethyl 8-chloro-7-oxooctanoate. The mixture was heated under a reflux for 8 hours and filtered. The xylene was removed *in vacuo* and the residual oil solidified when it was cooled for several hours at  $-10^{\circ}$ . This crude product, which weighed 11.5 gm., was recrystallized from ethanol-water. The crystalline material was collected by filtration and weighed 9.6 gm., 75 per cent of the theoretical yield from the ethyl  $\epsilon$ -chloroformyl caproate. The crude phthalimido product may be used for the subsequent step without recrystallization. For analysis, a small sample of the phthalimido ester was recrystallized from ethanol-water. The homologous phthalimido compounds were prepared in good yield by a similar procedure and their properties and analyses are tabulated in Table I.

*2,3-Dihydro-2-oxo-4-imidazolecaproic Acid (VII)*—The crude ethyl 8-phthalimido-7-oxooctanoate, obtained from 25.1 gm. of ethyl 8-chloro-7-oxooctanoate, was heated under a reflux for 12 hours with 400 cc. of 6 N HCl.

The hydrolysate was cooled overnight and the precipitated phthalic acid was removed by filtration. The filtrate, containing the 8-amino-7-oxooctanoic acid hydrochloride (VI) and HCl, was concentrated *in vacuo* three times from water to remove the excess HCl. The amino ketone thus prepared was not isolated in pure form. The residue was dissolved in 60 cc. of water and treated with Darco. A solution of 15 gm. of KCNO in 20 cc. of water was added to the almost colorless filtrate. The crystalline imidazolonecaproic acid separated almost immediately. The acidity of the KCNO reaction mixture was maintained at pH 4 to 5 by the addition of acetic acid, while the mixture was heated on the water bath for 30 minutes. After the mixture had been cooled for several hours, the crystals were collected by filtration and were washed several times with acetone. The yield of 2,3-dihydro-2-oxo-4-imidazolecaproic acid (VII) was 15.9 gm., 63 per cent of the theoretical amount from ethyl  $\epsilon$ -chloroformyl caproate.

TABLE I  
*Phthalimido Compounds*

Compound	Empirical formula	N analysis		M.p.
		Calculated	Found	
		percent	percent	°C.
Ethyl 7-phthalimido-6-oxoheptanoate . . .	C <sub>17</sub> H <sub>19</sub> O <sub>5</sub> N (317.3)	4.41	4.64	58-59
" 8-phthalimido-7-oxooctanoate . . . . .	C <sub>18</sub> H <sub>21</sub> O <sub>5</sub> N (331.4)	4.23	4.12	61-62
" 9-phthalimido-8-oxononoate . . . . .	C <sub>19</sub> H <sub>23</sub> O <sub>5</sub> N (345.4)	4.06	3.99	54-56
" 10-phthalimido-9-oxodecanoate . . . .	C <sub>20</sub> H <sub>25</sub> O <sub>5</sub> N (359.4)	3.90	4.01	64-65

The homologous imidazolone aliphatic acids were prepared in similar yield by the same procedure as that which is described for the synthesis of the imidazolonecaproic acid. These compounds were purified either by recrystallization from ethanol-water or by acidification of a warm NaHCO<sub>3</sub> solution of the acids. The melting points and elementary analyses of these imidazolone aliphatic acids are listed in Table II.

The ultraviolet absorption spectra of the various imidazolone aliphatic acids were determined by use of a Beckman spectrophotometer (model DU) and are plotted in Fig. 1. These compounds showed only end-absorption, in contrast to 2,3-dihydro-2-oxo-4-imidazolecarboxylic acid. The latter compound was prepared by the method described by Hilbert (12).

*dl*-2-Oxo-4-imidazolidinecaproic Acid (VIII)—15.9 gm. of 2,3-dihydro-2-oxo-4-imidazolecaproic acid were dissolved in 80 cc. of 5 per cent NaHCO<sub>3</sub> solution, and 3.0 gm. of solid NaHCO<sub>3</sub> were added to bring the pH of the mixture to 7.6. The solution was placed in a high pressure hydrogenation bomb and approximately 12 gm. of Raney's nickel catalyst were added. The hydrogenation was carried out under a hydrogen pressure of 2210

TABLE II  
*Imidazolone Aliphatic Acids*

Imidazolone Aliphatic Acids								
Compound	Empirical formula	Elementary analysis						M.p.
		C		H		N		
		Calculated	Found	Calculated	Found	Calculated	Found	
		per cent	per cent	per cent	per cent	per cent	per cent	°C.
2,3-Dihydro-2-oxo-4-imidazolevaleric acid	C <sub>8</sub> H <sub>12</sub> O <sub>3</sub> N <sub>2</sub> (184.2)	52.16	52.16	6.57	6.61	15.21	15.46	242-244
2,3-Dihydro-2-oxo-4-imidazolecaproic acid	C <sub>9</sub> H <sub>14</sub> O <sub>3</sub> N <sub>2</sub> (198.2)	54.53	54.10	7.12	6.94	14.14	13.74	178-179
2,3-Dihydro-2-oxo-4-imidazoleheptanoic acid	C <sub>10</sub> H <sub>16</sub> O <sub>3</sub> N <sub>2</sub> (212.3)	56.57	56.94	7.60	7.99	13.20	13.48	228-229
2,3-Dihydro-2-oxo-4-imidazoleoctanoic acid	C <sub>11</sub> H <sub>18</sub> O <sub>3</sub> N <sub>2</sub> (226.3)	58.38	58.30	8.02	7.97	12.38	12.01	169-170

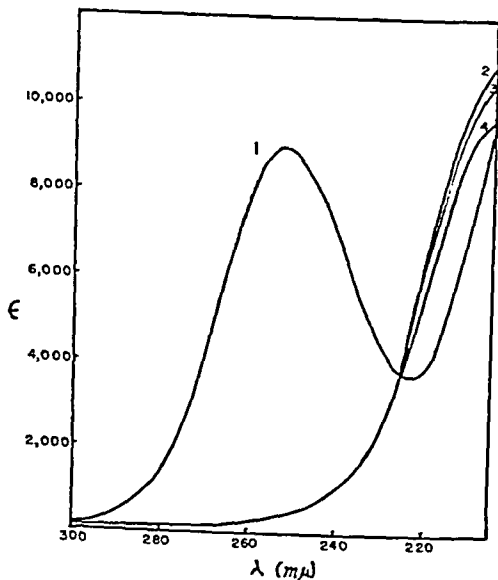


FIG. 1. Ultraviolet absorption spectra. Curve 1, 2,3-dihydro-2-oxo-4-imidazole-carboxylic acid; Curve 2, 2,3-dihydro-2-oxo-4-imidazolevaleric acid and 2,3-dihydro-2-oxo-4-imidazolecaproic acid; Curve 3, 2,3-dihydro-2-oxo-4-imidazoleheptanoic acid; and Curve 4, 2,3-dihydro-2-oxo-4-imidazoleoctanoic acid in water. The wavelength is plotted against the molecular extinction,  $\epsilon$ . ( $\epsilon = \frac{E \times \text{mol. wt.}}{cd}$  where  $E$  = extinction, mol. wt. = molecular weight of compound,  $c$  = concentration in gm. per liter, and  $d$  = cell thickness in cm.)

pounds at 100° with shaking for 24 hours. At the end of this period, the nickel was removed by filtration. Upon acidification of the filtrate with  $\text{H}_2\text{SO}_4$ , the *dl*-2-oxo-4-imidazolidinecaproic acid separated. After the mixture had been allowed to cool for several hours, the crystalline product was collected by filtration. The homologous imidazolidone aliphatic acids were prepared under comparable conditions from the corresponding imidazolone aliphatic acids. The properties and analyses of these imidazolidone aliphatic acids are recorded in Table III.

*Methyl dl*-2-Oxo-4-imidazolidinevalerate—5.8 gm. of 2,3-dihydro-2-oxo-4-imidazolevaleric acid were suspended in 100 cc. of absolute methanol. 7 gm. of dry  $\text{HCl}$  were passed into the mixture and it was heated under a reflux for 1 hour. After about half of the alcohol had been removed by distillation, 50 cc. of saturated  $\text{NaHCO}_3$  solution were added and the solution was rendered alkaline by the addition of solid  $\text{NaHCO}_3$ . The methyl ester of imidazolonevaleric acid was extracted with ethyl acetate. After the ethyl acetate solution had been dried over  $\text{Na}_2\text{SO}_4$ , the solvent was removed *in vacuo*. The residue of methyl 2,3-dihydro-2-oxo-4-imidazolevalerate was dissolved in 100 cc. of methanol containing 15 gm. of activated nickel on kieselguhr catalyst and hydrogenated according to the method described by Winans and Adkins (13). The hydrogenation was carried out for 6 hours at 200° under a hydrogen pressure of 2700 pounds. The catalyst was then removed and the filtrate was concentrated *in vacuo* to a green oil which was purified by sublimation *in vacuo*. The sublimate of methyl *dl*-2-oxo-4-imidazolidinevalerate was recrystallized from ether containing a few drops of methanol by cooling the solution at -13°. The crystalline material melted at 84.5–85°.

$\text{C}_9\text{H}_{15}\text{O}_5\text{N}_2$ .	Calculated.	C 53.98, H 8.06, N 14.00
200.2	Found.	" 54.01, " 8.26, " 13.92

Ethyl *dl*-2-oxo-4-imidazolidinevalerate was prepared by a procedure identical to that used for the methyl ester. The imidazolonevaleric acid was esterified with ethanol and  $\text{HCl}$ , and the hydrogenation was carried out in ethanol. The recrystallized ethyl *dl*-2-oxo-4-imidazolidinevalerate melted at 86–86.5°

$\text{C}_{10}\text{H}_{17}\text{O}_5\text{N}_2$ .	Calculated.	C 56.04, H 8.47
214.3	Found.	" 56.03, " 8.45

*Methyl dl*-2-Oxo-4-imidazolidinecaproate—2-Oxo-4-imidazolidinecaproic acid was esterified with methanol and  $\text{HCl}$  and the resulting ester was hydrogenated as described above. The sublimate of methyl *dl*-2-oxo-4-imidazolidinecaproate melted at 88–89° after recrystallization from methanol-ether.

$\text{C}_{10}\text{H}_{17}\text{O}_5\text{N}_2$ .	Calculated.	C 56.04, H 8.47, N 13.08
214.3	Found.	" 56.23, " 8.05, " 12.89

TABLE III  
Imidazolidone Aliphatic Acids

Compound	Empirical formula	Elementary analysis								Yield	Recrystallization solvent	M.p. °C.
		C		H		N						
		Calcu- lated	Found	Calcu- lated	Found	Calcu- lated	Found					
		per cent	per cent	per cent	per cent	per cent	per cent					
<i>dl</i> -2-Oxo-4-imidazolidinevaleric acid	$C_6H_{11}O_5N_2$ (186.2)	51.60	51.66	7.58	7.01	15.05	15.10	64	Acetone-water or methanol-ether	169 -170		
<i>dl</i> -2-Oxo-4-imidazolidinoproic acid	$C_5H_9O_5N_2$ (200.2)	53.99	53.80	8.06	8.06	13.99	14.06	81	Water or 90% acetone	141.5-145		
<i>dl</i> -2-Oxo-4-imidazolidineheptanoic acid	$C_{10}H_{17}O_5N_2$ (214.3)	56.04	56.46	8.47	8.53	13.07	12.86	81	Water	156 -158		
<i>dl</i> -2-Oxo-4-imidazolidinooctanoic acid	$C_{11}H_{19}O_5N_2$ (228.3)	57.87	58.00	8.83	8.80	12.27	12.17	70	"	151 -152		



The hydrogenation of the esters of the imidazolone aliphatic acids with the nickel on kieselguhr catalyst gave much poorer yields than the hydrogenation of the sodium salts of the acids with Raney's nickel catalyst.

*Saponification of Methyl dl-2-Oxo-4-imidazolidinevalerate and Methyl dl-2-Oxo-4-imidazolidinecaproate*—The methyl esters were saponified by mild treatment with dilute NaOH, since a preliminary experiment with ethyl *dl*-2-oxo-4-imidazolidinevalerate showed it to be cleaved to *dl*-6,7-diaminoheptanoic acid dihydrochloride by heating with 6 N HCl.

300 mg. of methyl *dl*-2-oxo-4-imidazolidinevalerate were treated with 21.2 cc. of 0.1 N NaOH for 15 minutes at room temperature. The alkali was exactly neutralized by addition of the calculated amount of 0.1 N H<sub>2</sub>SO<sub>4</sub>. The solution was then concentrated to dryness *in vacuo* and the residue was extracted with 40 cc. of boiling ethanol. Crystals of *dl*-2-oxo-4-imidazolidinevaleric acid separated when the ethanol solution was concentrated *in vacuo*. 78 mg. of this acid, m.p. 168–169°, were obtained after recrystallization from a small amount of water.

194 mg. of methyl *dl*-2-oxo-4-imidazolidinecaproate and 16.0 cc. of 0.1 N NaOH were warmed at 50° for 15 minutes. The alkali was exactly neutralized by addition of the calculated amount of 0.1 N H<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to a volume of about 10 cc. and cooled for several hours. 134 mg. of crystalline *dl*-2-oxo-4-imidazolidinecaproic acid separated. The product melted at 144–145° after recrystallization from a small amount of water.

*dl*-6,7-Diaminoheptanoic Acid Sulfate—A solution of 500 mg. of 2-oxo-4-imidazolidinevaleric acid in 5.0 cc. of 6 N H<sub>2</sub>SO<sub>4</sub> was heated for 2 hours at 120° in the autoclave and then cooled. Upon the addition of 10 cc. of methanol and 2 cc. of diethyl ether, the crystalline diaminoheptanoic acid sulfate separated. The yield after recrystallization from water-methanol was 571 mg. The *dl*-6,7-diaminoheptanoic acid sulfate melted with decomposition at 250–253°.

*Synthesis of dl-2-Oxo-4-imidazolidinevaleric Acid from dl-6,7-Diaminoheptanoic Acid Sulfate*—Phosgene gas was bubbled into a solution of 400 mg. of *dl*-6,7-diaminoheptanoic acid sulfate in 10 cc. of 10 per cent Na<sub>2</sub>CO<sub>3</sub> until the solution became acid to Congo red, according to the method described by Melville (14) for the synthesis of desthiobiotin from diamino-pelargonic acid. The mixture was concentrated *in vacuo* to a volume of 6 cc. This concentrate was then extracted continuously with ether for 60 hours. 137 mg. of *dl*-2-oxo-4-imidazolidinevaleric acid were thus obtained. Further extraction yielded no more product. 1.0 gm. of Na<sub>2</sub>CO<sub>3</sub> was added to the aqueous solution and the treatment with phosgene was repeated. Continuous extraction with ether yielded an additional 80 mg. of imidazolidonevaleric acid. A total yield of 217 mg. of the imidazolidone-

valeric acid, 75 per cent of the theoretical amount, was obtained. This compound melted at 166–167° after recrystallization from methanol-ether, and showed no depression of the melting point when it was mixed with a sample of the original *dl*-2-oxo-4-imidazolidinevaleric acid from which the diaminoheptanoic acid was prepared.

*Preparation of dl*-2-Oxo-4-imidazolidinecaproic Acid from *dl*-7,8-Diaminooctanoic Acid Sulfate—The treatment of 200 mg. of *dl*-2-oxo-4-imidazolidinecaproic acid with 2 cc. of 6 *N*  $\text{H}_2\text{SO}_4$  at 120° for 2 hours yielded 243 mg. of *dl*-7,8-diaminooctanoic acid sulfate. After recrystallization from water-methanol, this compound melted with decomposition at 258–261°.

50 mg. of the diaminooctanoic acid sulfate were dissolved in 5 cc. of 10 per cent  $\text{Na}_2\text{CO}_3$  and treated with phosgene until the solution was acid to Congo red. By continuous extraction of this solution with ether, 27 mg. of *dl*-2-oxo-4-imidazolidinecaproic acid were obtained. No depression of the melting point was observed when this product was mixed with the *dl*-imidazolidonecaproic acid from which the diaminoctanoic acid sulfate was prepared.

*dl*-2-Oxo-4-imidazolidinecarboxylic Acid—2,3-Dihydro-2-oxo-4-imidazolecarboxylic acid was prepared from tartaric acid and urea with  $\text{H}_2\text{SO}_4$ , as described by Hilbert (12). The acid was esterified with absolute ethanol and sulfuric acid. Several attempts to hydrogenate ethyl 2,3-dihydro-2-oxo-4-imidazolecarboxylate in the presence of nickel on kieselguhr catalyst gave a mixture of products which was difficult to purify.

Some *dl*-2-oxo-4-imidazolidinecarboxylic acid was obtained by reduction of the sodium salt of imidazolonecarboxylic acid in  $\text{NaHCO}_3$  solution with Raney's nickel catalyst, but decarboxylation also occurred, resulting in the formation of ethylene urea and other unidentified by-products.

20 gm. of 2,3-dihydro-2-oxo-4-imidazolecarboxylic acid were dissolved in 170 cc. of a 5 per cent solution of  $\text{NaHCO}_3$  and solid  $\text{NaHCO}_3$  was added until the pH of the solution was 7.6. Approximately 25 gm. of Raney's nickel catalyst were added and the mixture was hydrogenated for 16 hours at 100° under a pressure of 2200 pounds of hydrogen. The catalyst was removed by filtration. The filtrate was acidified to pH 4 with  $\text{H}_2\text{SO}_4$  and concentrated to dryness *in vacuo*. The residue was extracted with boiling ethanol. The alcohol was removed *in vacuo* and the residue was dissolved in hot water. A small amount of crystalline *dl*-2-oxo-4-imidazolidinecarboxylic acid separated when this aqueous solution was cooled.

The aqueous solution was rendered alkaline with  $\text{NaHCO}_3$  and extracted continuously with ethyl acetate. When this extract was cooled, 2.5 gm. of crystalline ethylene urea separated. The ethylene urea was recrystallized from ethyl acetate and melted at 131–132°. A mixed melting point with an authentic sample of ethylene urea showed no depression.

The alkaline solution was acidified and extracted continuously with ethyl acetate. Approximately 4 gm. of crude *dl*-2-oxo-4-imidazolidine carboxylic acid were obtained. The *dl*-2-oxo-4-imidazolidinecarboxylic acid melted at 199–201° after it was recrystallized twice from water.

$C_8H_9O_3N_2$ .	Calculated.	C 36.92,	H 4.65,	N 21.54
130.1	Found.	" 37.15,	" 4.85,	" 20.88

## SUMMARY

The synthesis of imidazolidonecaproic acid, which differs from desthiobiotin only by the absence of the terminal methyl group, has been described in detail. The syntheses and properties of imidazolidonevaleric, imidazolidoneheptanoic, and imidazolidoneoctanoic acids are also reported.

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# RELATIONSHIP OF THIOL STRUCTURES TO REACTION WITH ANTIBIOTICS

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A large number of the antibiotics have demonstrated chemical reactivity toward compounds containing sulfhydryl groups (1, 2). There have also been observed marked differences in reactivity of individual antibiotics toward various types of sulfhydryl-containing compounds. In the present investigation, the effect of structure of thiol compounds on their ability to react with certain antibacterial agents is interpreted in terms of the possible relationship of structure of thiol groups in proteins with factors of specificity and degree of activity of antibiotics. The importance of sulfhydryl groups to enzyme activity has been demonstrated frequently (3-9), although the exact functions of the group are still in doubt.

The antibiotics studied were penicillin G, streptomycin, gliotoxin, pyocyanine, and the active principles of *Allium sativum* ( $C_6H_{10}S_2O$ ), *Arctium minus* ( $C_{15}H_{20}O_8$ ), and *Asarum canadense* (principle A) (10). This is a chemically heterogeneous group with examples of antibiotics derived from bacteria, molds, *Actinomyces*, and higher plants, but all react with certain sulfhydryl compounds. Some of these antibiotics previously (2) were discussed briefly with respect to inactivation by a number of thiols and it was postulated that this class of antibacterial agents acted by combining with essential sulfhydryl groups of enzymes and that probable factors involved were diffusibility of the antibiotic throughout the microbial cell structure, degree of adsorption of the antibiotic by enzymes, and ability of the antibiotic to react with sulfhydryl groups of the enzymes.

Penicillin has shown a high degree of specificity in its reaction with thiols. Cysteine, its esters, and  $\beta$ -amino- and  $\beta$ -dimethylaminoethanethiols are good penicillin inactivators; homocysteine, N-acetylcysteine, and thioglycolate are poor (2). Cysteine and aminoethanethiol are equally effective; however, *dl*- $\beta$ , $\beta$ -dimethylcysteine (*dl*-penicillamine) (11) is not effective, showing interference from  $\beta$ -carbon substituents other than hydrogen. On the basis of present information the ideal thiol type of inactivator for penicillin should possess a basic amino group (primary through tertiary) on the carbon adjacent to the carbon carrying the sulfhydryl group, with substituents on the nitrogen smaller than ethyl (methyl or hydrogen). When using this information in predicting the possible

reactivity of penicillin toward enzyme —SH groups, one is limited to the consideration of only such thiol structures derived from known amino acids which might exist in protein molecules. The low reactivity of N-acetylcysteine as compared with that of cysteine and its esters may indicate that the cysteine unit in proteins would show greatest reactivity toward penicillin when its amino group is not acylated.

It was felt desirable to prepare sulfhydryl-containing peptides for comparison of reactivity toward the antibiotics, since cysteine esters and acetylcysteine are not true models of structures present in proteins. The two most simple dipeptides of cysteine, glycyl-L-cysteine and L-cysteinylglycine, were prepared. Cysteine, N-acetylcysteine, glutathione, thioglycolate, and S-methylcysteine were included for comparison of reactivity.

#### EXPERIMENTAL

*Glycyl-L-cysteine Hydrochloride*—The dipeptide salt was prepared by applying the procedure described by Greenstein (12) for the preparation of diglycyl-L-cystine. The residue from the sodium-liquid ammonia reduction was dissolved in water and acidified with hydrochloric acid, and a solution of mercuric chloride and sodium acetate was added to precipitate the mercaptide. The precipitate was washed thoroughly with water, suspended in water, and treated with hydrogen sulfide to liberate the dipeptide. After removal of excess hydrogen sulfide by distillation under reduced pressure, the solution was treated with a dilute solution of mercuric chloride and the precipitate again thoroughly washed with water. The dipeptide was again liberated by treatment of the mercaptide with hydrogen sulfide, the precipitate filtered off, and the solution concentrated under reduced pressure. The concentrate was made strongly acid with hydrochloric acid and was allowed to evaporate to dryness over solid sodium hydroxide in a vacuum desiccator. The hygroscopic, amorphous glycyl-L-cysteine hydrochloride (m.p., decomposition,  $>90^\circ$ ) was finally dried over phosphorus pentoxide. Grote's test for sulfhydryl groups (13) was positive. The rotation was  $[\alpha]_D^{25} = +2.5^\circ$  (20 mg. per cc. of water); rotation of L-cysteine hydrochloride in the same molar concentration (13.6 mg. per cc. of water) was  $[\alpha]_D^{25} = +8.1^\circ$ .

$C_4H_{11}O_2N_2SCl$ . Calculated.	N 13.05,	ionic Cl 16.52
Found.	" 12.85,	" " 17.20

*L-Cysteinylglycine Hydrochloride*—This dipeptide salt was prepared by the reduction procedure of Loring and du Vigneaud (14) for N,N'-dicarboxy-L-cystinylglycine. The hydrochloride was isolated by the procedure described for the previous preparation. The amorphous hygroscopic salt with a melting point, with decomposition, of  $>70^\circ$  gave

a positive Grote's test for sulphydryl groups. The rotation was  $[\alpha]_D^{25} = +21.5^\circ$  (20 mg. per cc. of water).

Found. N 12.93, ionic Cl 17.15

**Reaction of Thiols with Antibiotics**—The reactions were carried out in potassium phosphate buffers at pH 6, 7, and 8 at  $25^\circ$ . The rate of reaction of the thiols with the antibiotics (except pyocyanine) was measured by observing loss of activity of the antibiotic when tested by the routine cylinder plate method (against *Staphylococcus aureus*). The pyocyanine reaction was observed visually by the change in color from blue to green to colorless produced by the thiols. The thiol and antibiotic solutions were mixed and samples were removed for testing at intervals up to 24 hours. The thiol concentration in the reaction mixture was 5 mg. per cc. for cysteine and a corresponding molar concentration of the other thiols

TABLE I  
Effect of Thiol Structures on Rate of Inactivation of Antibiotics

Thiol	Approximate time required to inactivate			
	Penicillin G	Streptomycin	Asarum A	Pyocyanine*
	hrs.	hrs.	hrs.	hr.
<i>l</i> -Cysteine.....	<1	<0.25	4	0.25-0.5
<i>l</i> -Cysteinylglycine.....	<1	<0.25	4	0.25-0.5
Glycyl- <i>l</i> -cysteine.....	18	3	24	0.5
Glutathione.....	72	5	48	1
<i>N</i> -Acetylcysteine.....	>100	>100	>48	1
Thioglycolate.....	>>100	>100	>48	1

\* Reactivity measured by rate of color change.

was used. The concentrations of the antibiotics per cc. of reaction mixture were as follows: penicillin G and gliotoxin, 0.1 mg.; streptomycin, 200  $\gamma$ ;  $C_{15}H_{20}O_5$  (*Arctium*), pyocyanine, and  $C_6H_{10}S_2O$  (*Allium*), 1.0 mg.; and Asarum A, 0.05 mg. The streptomycin hydrochloride assayed approximately 800  $\gamma$  per mg.; the other antibiotics were crystalline preparations, except pure liquid  $C_6H_{10}S_2O$  (*Allium*).

In no instance did S-methylcysteine react in 24 hours with any of the antibiotics. Gliotoxin,  $C_6H_{10}S_2O$  (*Allium*), and  $C_{15}H_{20}O_5$  (*Arctium*) reacted with all six of the sulphydryl compounds within a few minutes reaction time. The approximate reaction time for complete inactivation of the other antibiotics is given in Table I. Penicillin and Asarum A showed an increase in rate of reaction with thiols with increase in pH from 6 to 7 to 8; the other antibiotics showed no appreciable differences with change of pH.

It is evident that in those instances in which an antibiotic shows specificity in its reaction with sulfhydryl groups the sulfhydryl on a non-acylated cysteinyl residue is the most reactive. The generally greater reactivity of glycylcysteine than acetylcysteine indicates that the free amino group in the former has some activating action but not nearly so great as that of a free amino group in the same cysteine residue.

On the basis of specificity and speed of reaction with thiols, the antibiotics may be divided as follows: Group I, those such as gliotoxin,  $C_6H_{10}S_2O$  (*Allium*), and  $C_{15}H_{26}O_6$  (*Arctium*), which are rapidly reactive toward most sulfhydryl compounds; Group II, intermediate ones such as pyrocyanine; which react with most sulfhydryl compounds, but with some differences in rate; and Group III, antibiotics such as penicillin, streptomycin, and *Asarum* A, which react slowly, with more specific sulfhydryl types and which show, with increase in concentration of the antibiotic, a marked increase in rate of reaction and lower molar ratios of thiol to antibiotic required for inactivation.

#### DISCUSSION

If we accept the proposal that this group of antibiotics inhibits growth by reacting with essential  $-SH$  groups in bacterial cells, two explanations for antibiotic action which are possible on the basis of the data are (a) the compounds may react with essential  $-SH$  groups of bacterial enzymes; (b) the compounds may react with the  $-SH$  groups in cysteinyl residues, as these are joined at the end of a growing polypeptide chain during protein anabolism. In this manner, antibiotics might block further growth of the protein along that chain by producing cysteine "dead ends."

Either or both of the mechanisms may be involved in inhibition of growth; however, with Group III antibiotics, reaction with enzyme  $-SH$  groups would be expected to take place readily only when the  $-SH$  is part of a cysteine residue attached at the end of a polypeptide chain, less readily with cysteine  $-SH$  in which free basic groups were in a neighboring amino acid of the chain, and least of all with a cysteine residue containing no neighboring basic amino groups. Group I antibiotics could react readily with almost any  $-SH$  group with which they can come in contact, the latter factor depending on diffusibility and adsorptive properties of the antibiotic. The higher antibacterial activity and specificity of a slowly  $-SH$ -reactive antibiotic such as penicillin as compared with rapidly reactive  $C_6H_{10}S_2O$  (*Allium*) might be explained partly on the basis of greater adsorbability of penicillin on enzymes and partly by "loss" of  $C_6H_{10}S_2O$  (*Allium*) by reaction with non-essential  $-SH$  groups of structural proteins, whereas penicillin would be "lost" primarily to essential  $-SH$ .

Penicillin, *Asarum* A, streptomycin, and pyocyanine react with cysteine at appreciable rates only in fairly high concentrations of reactants, whereas  $C_6H_{10}S_2O$  (*Allium*), gliotoxin, and  $C_{15}H_{20}O_5$  (*Arctium*) react readily even in low concentrations even though the former group includes some of the most active antibiotics. In order to account for this, it has been postulated that the more potent antibiotics are readily adsorbed in the vicinity of enzyme sulfhydryl groups. Differential adsorbability on bacterial enzymes of normal bacterial metabolites and certain bacteriostatic agents has been used by numerous investigators to explain the inhibiting action of such agents. In the inhibition of succinic dehydrogenase by malonate, Potter and DuBois (15) postulate specifically that the group blocked by the adsorbed interfering agent is the sulfhydryl and, in the case of quinonoidal compounds, a chemical reaction was proposed involving  $-SH$  and the quinone. There may be at least three types of inhibitors which can block  $-SH$  groups. (a) One type may be adsorbed by groups (such as  $-NH_2$  or  $-COOH$ ) in the vicinity of the  $-SH$  in such a manner as to block the  $-SH$  physically. (b) A second type may involve adsorption in the vicinity of the  $-SH$  groups, followed by a chemical reaction with the group (as possibly the quinones, penicillin, streptomycin, pyocyanine, and *Asarum* A). With unsaturated lactones, the possibility of a secondary reaction with amino groups also exists (16). (c) The third type may react rapidly with  $-SH$  with little or no selective adsorption prior to the reaction (possibly  $C_6H_{10}S_2O$  (*Allium*),  $C_{15}H_{20}O_5$  (*Arctium*),  $HgCl_2$ , and inorganic oxidizing agents).

The same forces in enzymes which attract the normally involved metabolite to the active  $-SH$  groups may be the forces attracting the inhibitor or antibiotic.

#### SUMMARY

The reactivity of six sulfhydryl compounds including glycyl-L-cysteine and L-cysteinylglycine toward seven antibiotics has been investigated. Gliotoxin and the active principles of *Allium sativum* and of *Arctium minus* show little specificity in reactivity toward thiols, whereas penicillin, streptomycin, and the antibiotic from *Asarum canadense* react much more readily with sulfhydryl compounds containing basic amino groups in the vicinity of the  $-SH$ . Pyocyanine has intermediate properties. The possible significance of the observations to the mechanism of interference of antibiotics with biologically essential  $-SH$  groups is discussed.

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## 2-KETO-D-GLUCONIC ACID IN THE POLYSACCHARIDE OF IRISH MOSS

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Numerous attempts have been made to isolate and characterize the polysaccharide extractable from Irish moss (*Chondrus crispus*). This red alga occurs in considerable quantities on the shores of Massachusetts, Maine, New Brunswick, Nova Scotia, and Prince Edward Island. It has recently been used commercially as a substitute for agar and in the manufacture of certain foods and pharmaceuticals because of its gelling and stabilizing properties.

The polysaccharide was first isolated by Schmidt (1) in 1844 and later given the name carrageenin by Stanford (2). This product has subsequently been referred to as Irish moss mucilage, carrageen or carrageen, and gelose. Recently Tseng (3) has made a commendable attempt to clarify the nomenclature of the polysaccharides found in seaweeds and we shall follow his suggestion of retaining the term carrageenin for this polysaccharide. In 1868 Flückiger and Obermaier (4) claimed to have proved the presence of galactose, and in 1887 Hädecke, Bauer, and Tollens (5) the occurrence of fructose, galactose, and raffinose. Sebor (6) confirmed the presence of galactose and fructose but doubted that raffinose was present. He also claimed to have found small quantities of a pentose and of glucose. The latter sugar has been detected by Muther and Tollens (7), Haas and Russell-Wells (8), and recently by Buchanan, Percival, and Percival (9).

That carrageenin is an ethereal sulfate was first shown by Haas and Hill (10), and the nature of this ester has been the subject of several investigations (9, 11-15). These investigations may be summarized by stating that carrageenin is probably an  $\alpha$ -1,3-galactopyranoside 4-sulfate, occurring in nature as an acid salt combined mainly with calcium, potassium, and sodium, but also with small amounts of magnesium and ammonia. Galactose makes up 31 to 33 per cent of the molecule and the ash content is about 20 per cent. Buchanan, Percival, and Percival (9) present evidence from methylation studies to suggest the presence of a small amount of glucose, 1 to 2 per cent of pentose, and about 20 per cent of a ketose. It is thus apparent that there is a considerable portion of the polysaccharide as yet unidentified. Furthermore, all investigators have had difficulty with the purification of this polysaccharide to the point of chemical indi-

viduality, and there is no convincing proof of the presence of only one polysaccharide in the preparations studied. Indeed, Haas and his colleagues have separated two fractions, a cold water extract and a hot water extract of the plant. From our own experience with the behavior of these two kinds of extract towards acid at pH 4 to 5, we are not convinced that this is a true separation. We have been able to extract all of the carrageenin from the alga by prolonged treatment with cold water. The colloidal aggregates are polydisperse and the ionic balance will change from the initial equilibrium in the cells with prolonged extraction. These physico-chemical studies have been published recently (16).

The object of the present investigation was to attempt to determine the nature of the unknown fraction of the carrageenin molecule. We have been successful in isolating 2-keto-D-gluconic acid (17). This compound is referred to as fructuronic acid by English chemists.

#### EXPERIMENTAL

*Extraction of Polysaccharide*—Fresh Irish moss was collected on Prince Edward Island, dried and bleached by exposure to sunlight outdoors, roughly ground, and stored. This material was extracted (100 ml. to 1 gm.) with boiling water for 1 to 2 hours, filtered through fine cheesecloth, and then through a Whatman No. 3 paper covered with Hyflo Super-Cel filter aid (Johns-Manville) or Celite on a Büchner funnel. The filtrate was concentrated *in vacuo* at 30–40° to a viscous residue and then poured into 4 volumes of 95 per cent ethanol. The product was dissolved in water and dialyzed for 6 days in a modified Sørensen dialyzer with vacuum control. The residual solution was concentrated to partial dryness *in vacuo* at 30–40° and finally dried at 60° over phosphorus pentoxide in a "pistol" desiccator.

The product was a fine amorphous, hygroscopic powder, usually white but sometimes showing a faint yellow color. It contained 18.4 per cent ash and 0.2 per cent of nitrogen, as estimated by Pregl's micro-Kjeldahl method. Calcium and sulfate in the ash were found to be 4.19 and 13.6 per cent respectively of the dry polysaccharide, and together accounted for about 97 per cent of the ash.

*Hydrolysis by Hydrochloric Acid*—4.25 gm. of carrageenin were boiled under a reflux with 200 ml. of 2 per cent HCl for 14 hours. A dark brown color developed. The solution was treated with solid silver carbonate to remove chloride. Excess of silver was removed by hydrogen sulfide and the filtrate was slightly acidified with acetic acid and concentrated to 50 ml., when 60 ml. of 95 per cent ethanol were added. A small precipitate formed which was not identified. The filtrate was evaporated to dryness on the steam bath. On extracting the residue with ethanol and evaporating the

extract, brownish leaf-like crystals separated, which gave an indefinite melting point.

These, on treatment with phenylhydrazine, yielded a crystalline product which melted at  $164^{\circ}$ . This product, on being warmed with pyridine and benzoyl chloride, yielded crystals which melted at  $121^{\circ}$ . The former compound corresponds in properties with *dl*-arabinosazone, m.p.  $166$ – $168^{\circ}$ , and the latter with the tribenzoyl ester of arabinolactone, m.p.  $120^{\circ}$  (18).

*Hydrolysis by Oxalic Acid*—2 gm. of carrageenin were boiled under a reflux in an atmosphere of nitrogen for 30 hours in 200 ml. of an aqueous solution of oxalic acid and potassium oxalate at a concentration of 0.05 *M*. Under these conditions, the solution became only slightly yellow. The course of the hydrolysis was followed by estimations of (a) free sulfate gravimetrically as  $\text{BaSO}_4$ , (b) the total reducing power by the method of Folin and Wu (19), and (c) ketose by the orcinol reagent (20). The curves for all three estimations were parallel and attained maximal values in about 24 hours.

The hydrolysis was repeated with 10 gm. of carrageenin. The resulting solution was decolorized with charcoal (norit A), filtered, and chilled overnight at  $4^{\circ}$ . The filtrate was neutralized with KOH and evaporated to dryness *in vacuo* at  $30$ – $40^{\circ}$ . The brown syrupy residue was extracted with hot ethanol (95 per cent) until no further color was extractable. The combined extracts were evaporated to dryness *in vacuo* at  $30^{\circ}$ , the residue being redissolved in absolute ethanol and the process repeated several times. Finally the residue was dried over  $\text{P}_2\text{O}_5$  in a "pistol" desiccator.

*Color Reactions*—The usual color reactions were carried out on a few mg. of the alcohol-soluble residue with the following results: Benedict, positive; Molisch, purple; phloroglucinol, amber; orcinol, brownish; naphthoresorcinol, red; Dische, negative. The alcohol-insoluble material gave positive reactions with the Benedict, Molisch, and phloroglucinol tests but negative reactions with the other reagents. The reactions given by calcium 2-ketogluconate, isolated later, were found to be as follows: Benedict, positive; Molisch, purple; phloroglucinol, greenish yellow; orcinol, green on prolonged heating only; naphthoresorcinol, brownish pink; Dische, negative; resorcinol, green on prolonged heating.

*Isolation of 2-Ketogluconic Acid As Diisopropylidene Derivative*—The whole of the alcohol-soluble residue, 1.90 gm., was suspended in 500 ml. of absolute acetone, together with 40 gm. of anhydrous  $\text{CuSO}_4$ . The flask was shaken occasionally for 5 days at  $20^{\circ}$ . The solution was then filtered and concentrated *in vacuo* at 1 cm. pressure to a light yellow oil which crystallized on standing. The product was suspended in 150 ml. of ether and 10 ml. of 0.01 *N*  $\text{H}_2\text{SO}_4$  were added. The ether layer was dehydrated with anhydrous  $\text{Na}_2\text{SO}_4$  and allowed to evaporate. The oil obtained was dis-

tilled fractionally at a pressure of 0.1 mm. The fraction collected up to 175° crystallized on standing, and was recrystallized from a mixture of ether and petroleum ether. The melting point was 95° and  $[\alpha]_D^{22} = -48.8^\circ$ . Microelementary analysis showed C 52.99 per cent, and H 6.81 per cent. Constants for diisopropylidene-2-ketogluconic acid have been recorded as m.p. 96–97°,  $[\alpha]_D^{22} = -49.38^\circ$  (21), and values for C 52.72, H 6.57 per cent, calculated.

*Synthesis of 2-Ketogluconic Acid*—A small quantity of diisopropylidene-2-ketogluconic acid was prepared from fructose by oxidation with alkaline permanganate, according to the procedure of Ohle and Wolter (21). A mixed melting point determination with our product from carrageenin gave the same value, 95°, as was obtained previously.

*Preparation of Anilide*—500 mg. of the diacetone derivative from carrageenin were dissolved in 2 ml. of 10 per cent aqueous KOH and allowed to stand overnight. The solution was evaporated and the residue was taken up in absolute ethanol. To this were added 400 mg. of aniline hydrochloride in absolute ethanol. After filtration from the precipitated KCl, the filtrate was decolorized with charcoal and concentrated to crystallization; m.p. 122°,  $[\alpha]_D^{22} = -28.9^\circ$ ; previously recorded values for the anilide, m.p. 120°,  $[\alpha]_D^{20} = -31.3^\circ$  (21).

*Preparation of Methyl Ester*—270 mg. of the original crystalline product were dissolved in 10 ml. of absolute ether and treated with 210 mg. of  $\text{PCl}_5$ . After several hours at 4°, 3 ml. of 3 per cent sodium methoxide in methanol were added. The solution was decolorized with charcoal and evaporated to crystallization; m.p. 52°,  $[\alpha]_D^{22} = -43.9^\circ$ ; recorded values for the methyl ester, m.p. 52°,  $[\alpha]_D^{22} = -44.7^\circ$  (21).

*Identification of Galactose in Alcohol-Insoluble Residue*—The residue after the hydrolysis of carrageenin with oxalic acid and subsequent extraction with hot ethanol weighed 13.2 gm. This material was dissolved in the minimum volume of water and the oxalate ion was precipitated with excess  $\text{CaCl}_2$ . The filtrate was methylated with dimethyl sulfate and 8 N NaOH, according to the procedure of Haworth (22). On extraction with chloroform, a syrup was obtained which distilled at 150–155° and 0.1 mm. pressure. This behavior corresponds with that of trimethylmethylgalactoside and identifies the presence of galactose in carrageenin, as has been reported previously by several investigators.

*Methylation of Carrageenin*—20 gm. of the purified polysaccharide were methylated according to the method of Haworth (22) by dissolving it in 200 ml. of water and treating the solution with dimethyl sulfate and sodium hydroxide at 60–80° for 24 hours. The reagents were added in small portions over 2 hours with vigorous stirring. The resulting solution was dialyzed against tap water until free from sulfate and the residual solution

was concentrated *in vacuo* at 40–50°. The process of methylation was repeated. Hydrolysis was next carried out in 100 ml. of 0.05 M oxalic acid and potassium oxalate under nitrogen. On cooling the solution to 0°, most of the oxalate crystallized out. The mother liquor was concentrated to 50 ml. *in vacuo* at 30–40°, and more oxalate was removed, and finally taken to dryness. The residue was dissolved in ethanol, and the solution concentrated. Water was added and the solution was transferred to a special 1 liter flask equipped with a stirring apparatus and sealed ground glass joints. Methylation, as previously described, was repeated. The solution was extracted with chloroform as solvent for the methylated product, which facilitated separation from the  $\text{Na}_2\text{SO}_4$ . This procedure of methylation was repeated twice more. Finally, the residue was extracted with chloroform and again methylated with methyl iodide and silver oxide, according to the procedure of Purdie and Irvine (23). The chloroform was distilled off *in vacuo* at 40° and 16 to 20 mm. pressure and the following four fractions were obtained at 0.05 mm. pressure.

*Fraction 1*—1.2 gm. of a colorless liquid distilled between 40–60°. This fraction reduced Fehling's solution after hydrolysis with 0.5 per cent HCl for 6 hours at 80°. It gave a crystalline semicarbazone, m.p. 166–167°, and an oxime, m.p. 102–104°. The product was tentatively identified as  $\omega$ -methoxy-5-methylfurfural, which forms a semicarbazone with a melting point of 166–167° and an oxime with a melting point of 103–104° (24).

*Fraction 2*—2.2 gm. of an oil were obtained between 60–110°, which crystallized as colorless needles, m.p. 94–95°. The material was soluble in ether, petroleum ether, acetone, and ethanol. It was not identified.

*Fraction 3*—5.3 gm. of a light brown oil distilled between 110–140°. This fraction was soluble in ether but failed to crystallize. It did not reduce Fehling's solution until after hydrolysis with 2 per cent sulfuric acid for 12 hours at 80°. Evaporation of the neutralized hydrolysate *in vacuo* at 40–45° and extraction with chloroform yielded a syrup which crystallized with a melting point of 70–73°, methoxyl content 56.5 per cent, and  $[\alpha]_D^{20} = +116.5^\circ$ . The anilide melted at 193°. These values correspond with 2,3,4,6-tetramethylgalactose and a determination of the mixed melting point confirmed the identification.

*Fraction 4*—1.1 gm. of an oil were obtained between 140–165°. This did not possess reducing properties until after hydrolysis. The methoxyl content was 56.4 per cent. Treatment with methanolic ammonia formed an amide, m.p. 96–98°. This corresponds with the amide of tetramethyl-2-ketogluconic acid and this was confirmed by a mixed melting point.

*Fraction 5*—No further distillate was obtained up to 250° and the residue in the flask was dark colored and vitreous in character.

*Isolation of Crystalline Methylated Carrageenin*—15 gm. of carrageenin in

200 ml. of distilled water were methylated as previously described by five successive treatments with 100 gm. of dimethyl sulfate. Between treatments the solution was neutralized with sulfuric acid, and sodium sulfate was removed by dialysis for 6 hours. After the final treatment, the solution was dialyzed for 6 days against running water at pH 6 and a temperature of 4°. The residual solution was centrifuged and concentrated to 50 ml. *in vacuo* at 40–50°. It was poured into 2 liters of 95 per cent ethanol. The precipitate was removed and the solution again concentrated to 50 ml. *in vacuo* at 20–30°. It was poured into 50 ml. of absolute ethanol and 150 ml. of ether were added. A copious white precipitate formed, which was allowed to stand for 5 days at 4°. The liquid was then filled with rosettes of microscopic needles (Fig. 1). These were filtered and analyzed;



FIG. 1. Crystals of methylated carrageenin.  $\times 70$

m.p. 130–140° with charring,  $[\alpha]_D^{18} = +48.0^\circ$ , methoxyl 15.2 per cent, ash 18.2 per cent. Similar values obtained on amorphous methylated carrageenin were m.p. 130–140° with charring,  $[\alpha]_D^{18} = +47.6^\circ$ , methoxyl 14.8 per cent, ash 17.9 per cent.

*Attempted Estimation of 2-Ketogluconic Acid; Resorcinol Reaction*—This determination was first attempted by the technique of the resorcinol reaction (25). A control solution of 0.5 per cent calcium ketogluconate showed no color in 20 minutes and only a light green after 1 hour at 80°. Two preparations of the polysaccharide were submitted to the same conditions and compared to a standard solution of 0.010 per cent fructose. Calculated as fructose on a moisture-free basis, the cold extract of Irish moss gave a titer of 14.8 per cent and the hot extract, 22.4 per cent. The error introduced by galactose at a concentration of 35 per cent of the carrageenin

was found to be 7.15 per cent of the color obtained. Thus the corrected values for ketose in the cold extract would be 13.7 per cent and in the hot extract 20.8 per cent. Under the conditions of the determination, longer heating did not change the values obtained. So high a proportion of ketose is somewhat surprising because of our inability to isolate the corresponding diisopropylene derivative or the osazone.

*Naphthoresorcinol Reaction*—The estimation was carried out according to the technique of Kapp (26). The standard color was developed in a tube containing 0.455 mg. of ketogluconic acid as the calcium salt. The color developed slowly and was not evident within 1 hour, thereby resembling the behavior of galacturonic acid. The color was compared in a photocolormeter at wave-lengths of 550 and 590  $m\mu$  with that given by 1 mg. of carrageenin. The result indicated more than 100 per cent of ketogluconic acid in the polysaccharide. It was concluded that other constituents participated in the reaction and yielded color with greater intensity than the standard used. The method is therefore inapplicable.

*Furfural Phloroglucide Reaction*—Lefèvre and Tollens (27) have shown that glucuronic acid will form furfural to the extent of one-third of its weight as phloroglucide under the conditions for the estimation of pentose. After refluxing a solution of 0.388 gm. of ketogluconic acid in 12 per cent HCl for 4 hours and distilling according to the Kröber procedure (28), 0.130 gm. of phloroglucide was obtained. This corresponds to exactly one-third of the original acid. This procedure, applied to 1.314 gm. of carrageenin after 7 hours refluxing, formed only 0.0143 gm. of phloroglucide, the equivalent of 3.26 per cent of ketogluconic acid. If calculated as pentose, the result corresponds to 0.98 per cent, which is in agreement with the figure of 1 per cent obtained by Buchanan, Percival, and Percival (9).

#### DISCUSSION

The finding of 2-keto-D-gluconic acid as a product of the hydrolysis of carrageenin is the first recorded instance of this substance as a constituent of a polysaccharide. It has been prepared synthetically by oxidation of glucose, of fructose, and of D-glucosone, and has been produced through the biochemical oxidation of glucose by *Acetobacter suboxydans* (29) and by various species of *Pseudomonas* (30). The substance is accordingly of biological interest. The finding of this sugar acid in the polysaccharide of Irish moss relates carrageenin with pectin, heparin, hyaluronic acid, and alginic acid in addition to its relationship, as an ethereal sulfate, with agar and with the polysaccharide from *Iridaea laminarioides* of Hassid.

Neither the free acid nor the lactone has been obtained crystalline in the free state and the substance must accordingly be studied in the form of



derivatives. During the initial hydrolysis with hydrochloric acid in the presence of air, we obtained some evidence for the formation of arabinose and arabonic acid as the osazone and benzoyl ester respectively. Decarboxylation of 2-ketogluconic acid would yield arabinose, and oxidation of the latter would form arabonic acid or arabinolactone. The formation of this last compound by acid oxidation has already been observed by Ohle and Berend (31); so that hydrolysis of carrageenin with hydrochloric acid in the presence of air might conceivably give rise to the products found. These findings throw some doubt on the claim to the existence of a pentose in carrageenin, unless further proof is advanced. This view is confirmed by the quantitative results obtained in the formation of the furfural phloroglucide from ketogluconic acid.

#### SUMMARY

From the products of hydrolysis of a purified sample of carrageenin, the polysaccharide of Irish moss (*Chondrus crispus*), with oxalic acid under nitrogen, 2-keto-D-gluconic acid has been isolated as the diisopropylidene derivative and thoroughly identified. 2-Keto-D-gluconic acid was found to give the color reactions of fructose except with the resorcinol test, and to form furfural in boiling 12 per cent HCl.

Further evidence for the presence of this compound was secured by the isolation of the amide of the tetramethyl derivative after exhaustive methylation of the polysaccharide, both before and after hydrolysis. The methylated polysaccharide was obtained in crystalline form and showed the following characteristics: m.p. 130–140° with decomposition,  $[\alpha]_D^{18} = +48.0^\circ$ , methoxyl 15.2 per cent, ash 18.2 per cent.

By hydrolysis of carrageenin with hydrochloric acid in air, some evidence was found for the formation of arabinose and arabinolactone as probable decomposition products of 2-ketogluconic acid.

The content of fructose in the polysaccharide obtained by cold extraction was 13.7 per cent and by hot extraction 20.8 per cent, as evidenced by the resorcinol reaction. The furfural phloroglucide obtained corresponded to 3.26 per cent of 2-keto-D-gluconic acid in carrageenin.

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# THE IN VIVO INACTIVATION BY CYANIDE OF BRAIN CYTOCHROME OXIDASE AND ITS EFFECT ON GLYCOLYSIS AND ON THE HIGH ENERGY PHOSPHORUS COMPOUNDS IN BRAIN

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The symptomatology of cyanide poisoning has been known for well over 100 years. Claude Bernard (1) was among the first to stress the inhibitory effect on the respiration of higher animals. The work of Keilin (2) and of Stotz (3) has shown that cyanide ion combines *in vitro* with cytochrome oxidase and thereby interferes with the utilization of molecular oxygen by the tissue oxidation-reduction systems. It has also been demonstrated that the utilization of molecular oxygen is coupled with phosphorylation reactions (4-7), and Lipmann (8) has emphasized the rôle of aerobic metabolism in the resynthesis of high energy phosphorus compounds. LePage<sup>1</sup> analyzed the tissues of rats subjected to experimental shock in a Noble-Collip rotating drum and observed elevated lactic acid and inorganic phosphate, low glycogen, adenosine triphosphate depletion, some phosphocreatine depletion, and an abnormal accumulation of phosphopyruvate. Proger, Decaneas, and Schmidt (9) have recently found that the readily hydrolyzable phosphorus fraction of kidney and heart tissue was decreased when rats were exposed to low oxygen tensions. These results indicate that in the intact animal also the resynthesis of high energy phosphorus compounds is coupled with oxidative processes.

The present experiments were undertaken, first, to determine whether brain tissue from cyanide-poisoned rats showed a diminution in cytochrome oxidase activity and, secondly, to study in some detail the distribution of glycogen, lactic acid, and phosphorylated intermediates in such tissue, particularly with reference to the distribution pattern of high energy phosphorus compounds.

## EXPERIMENTAL

*Measurement of Brain Cytochrome Oxidase Activity after Cyanide Injection*—A dose of 5 mg. per kilo of NaCN in aqueous solution was injected intraperitoneally into each of a series of adult male rats ranging in weight from about 250 to 400 gm. Rats so injected showed a rapid respiratory

<sup>1</sup> LePage, G. A., personal communication.

stimulation, followed by agitation, incoordination, convulsions, and ultimate cessation of respiration. At time intervals varying from  $3\frac{1}{2}$  to 8 minutes after injection, when respiration had just ceased, the animals were decapitated and the brain quickly removed and weighed on a torsion balance. The brain was then dropped into a Waring blender and enough iced, distilled water was added to make a 2 per cent final homogenate. The tissue was then homogenized for 2 minutes and strained through several layers of gauze to remove gross particles. Immediately before use, each homogenate was diluted with an equal volume of iced, distilled water. Control animals not injected with cyanide were treated in a similar fashion. Cytochrome oxidase activity was measured spectrophotometrically, as previously described.<sup>2</sup>

*Preparation of Brain Tissue for Analysis*—The methods and scheme of LePage and Umbreit (10) were followed in preparing the brain tissue for analysis and in analyzing for the various phosphorylated components. To minimize changes in the labile components attendant upon the sacrifice of the rats, the control or experimental animal was anesthetized by intraperitoneal injection of 50 mg. of pentobarbital per kilo, and immersed in liquid nitrogen.

The control animals were placed in liquid nitrogen 14 minutes after the injection of pentobarbital, when most of them were in surgical anesthesia; rats showing movement on immersion were not used. In the cyanide-poisoned animals, 5 mg. of NaCN per kilo were injected intraperitoneally 10 minutes after the administration of pentobarbital. These cyanide poisoned, anesthetized rats, in contrast to the cyanide-injected, unanesthetized animals, did not show any convulsions. Observations on a group of rats treated in this manner had shown that almost immediately after the administration of cyanide they exhibited a respiratory stimulation, followed within  $1\frac{1}{2}$  to 2 minutes by the onset of apnea of about  $1\frac{1}{2}$  to 2 minutes duration. The apnea was succeeded by gasping, irregular breathing, cessation of respiration, and death at about 8 to 13 minutes after the injection of cyanide, or about 18 to 23 minutes after the injection of pentobarbital. The rats were allowed to remain in the liquid nitrogen for  $1\frac{1}{2}$  to 2 minutes, at the end of which time they were removed and decapitated with a sharp axe.

*Analyses of Chemical Components*—The brain was extracted in trichloroacetic acid, according to the procedure of LePage and Umbreit (10). The trichloroacetic acid extract was quickly neutralized to pH 8.2 and used for the determination of the following constituents: lactic acid, inorganic phosphate, phosphocreatine, phosphopyruvic acid, and total acid-soluble phosphorus. The tissue residue remaining after extraction was used for

<sup>2</sup> Albaum, H. G., Tepperman, J., and Bodansky, O., *J. Biol. Chem.*, **163**, 641 (1946).

determination of glycogen. The procedures used were in accordance with those described in the analytical scheme of LePage and Umbreit (10), with two exceptions. In the determination of phosphopyruvate, no correction was made for the small amount of triose phosphate released in alkaline solution. The method used for the determination of glucose was that of Nelson (11).

The fractionation of the neutralized trichloroacetic acid filtrate was also carried out in accordance with the analytical scheme of LePage and Umbreit (10). To this filtrate were added a 25 per cent solution of barium acetate (0.05 ml. per mg. of phosphorus) and 4 volumes of cold 95 per cent ethanol. After standing overnight in the refrigerator, the mixture was centrifuged. The supernatant fluid (*alcohol-soluble fraction*) was poured off. The precipitate was dissolved in a few drops of  $N$  HCl, several ml. of water were added, and the pH was adjusted to 8.2 with  $2 N$  NaOH. The resulting suspension was chilled for 20 minutes and then centrifuged. The supernatant fluid (*alcohol-insoluble, barium-soluble fraction*) was poured off. The precipitate (*barium-insoluble fraction*) was dissolved in a few drops of  $N$  HCl, several ml. of water were added, and the barium was removed by precipitation with  $10 N$   $H_2SO_4$ . The supernatant fluid was decanted, the barium sulfate precipitate washed once with water and centrifuged, and the washings added to the supernatant fluid from the first centrifugation. The combined supernatants, representing the *barium-insoluble fraction*, were neutralized and made up to volume. The first fraction (*alcohol-soluble fraction*) contains compounds of negligible interest and hence was not analyzed in the present work. The second fraction (*alcohol-insoluble, barium-soluble fraction*) contains, among other phosphorylated intermediates, phosphocreatine and phosphopyruvate. However, since these compounds had been determined in aliquots from the neutralized trichloroacetic acid filtrate before treatment with barium acetate, this second fraction was not subjected to analysis. The third fraction (*barium-insoluble fraction*) contains inorganic phosphate, adenosine triphosphate, adenosine diphosphate, hexose diphosphate, and phosphoglycerate; these compounds were determined in accordance with LePage and Umbreit's scheme (10). All three fractions were analyzed for total phosphorus as a measure of the fractionation procedure. In the various experiments carried out, the sum of the total phosphorus of these three fractions ranged from 92 to 99 per cent of the total phosphorus in the original trichloroacetic acid filtrate.

### Results

*Cytochrome Oxidase Activity of Brain after Cyanide Injection*—Table I shows that the average cytochrome oxidase activity of brain, as measured

by the monomolecular reaction constant for the oxidation of cytochrome c, was  $0.243 \times 10^{-8}$  for nine normal rats. The average activity for the brains from six rats poisoned with NaCN was  $0.113 \times 10^{-8}$ . There was thus an average decrease of 54 per cent in the cytochrome oxidase activity in the brain.

*Anaerobic Glycolysis and High Energy Phosphorus Compounds after Cyanide Injection*—In Table II are shown the results of four separate experiments, each carried out with two control rats and two rats injected with NaCN. The concentrations of lactic acid, hexose diphosphate, phosphoglycerate, and phosphopyruvate were increased in the brains of

TABLE I

*Cytochrome Oxidase Activity of Brain Homogenates from Control Animals and Animals Injected Intraperitoneally with NaCN (5 Mg. per Kilo)*

The final reaction mixture had a volume of 3 ml. and contained 0.04 ml. of 1 per cent tissue homogenate, 0.01 M phosphate buffer, pH 7.4, and  $2.31 \times 10^{-5}$  M cytochrome c.

Control rats		Cyanide-injected rats	
Animal No.	Monomolecular reaction constant ( $K \times 10^8$ )	Animal No.	Monomolecular reaction constant ( $K \times 10^8$ )
	<i>min.<sup>-1</sup></i>		<i>min.<sup>-1</sup></i>
1	0.271	10	0.118
2	0.275	11	0.112
3	0.210	12	0.110
4	0.190	13	0.120
5	0.255	14	0.105
6	0.282	15	0.115
7	0.258		
8	0.240		
9	0.210		
Average:.....	0.243		0.113

the cyanide-poisoned animals. These average increases were marked in the case of lactic acid and phosphopyruvate. Although the changes were more variable for phosphoglycerate and hexose diphosphate, statistical treatment showed that the concentrations of these substances were also significantly increased. The concentration of glycogen in the brain of the cyanide-poisoned animals was significantly and markedly decreased. All these changes indicate quite clearly that in the rat brain, where the cytochrome oxidase has been partially inactivated by cyanide, there is a marked increase in glycolysis.

In contrast to the increase in the intermediaries in the glycolytic chain, the two high energy phosphorylated intermediaries, adenosine triphos-

TABLE II  
*Concentrations of Glycogen, Lactic Acid, and Phosphorus Compounds in Brains of Normal and Cyanide-Poisoned Rats*  
 Glycogen as mg. per 100 gm. of tissue. Inorganic phosphate as mg. of P per 100 gm. of tissue. All other values are calculated as mg. of acid per 100 gm. of tissue.

Phosphocreatine		Inorganic P		Lactic acid		Glycogen		Hexose diphosphate		Adenosine triphosphate		Adenosine diphosphate		Phosphoglycerate		Phosphopyruvate	
Control	Cy-anide	Control	Cy-anide	Control	Cy-anide	Control	Cy-anide	Control	Cy-anide	Control	Cy-anide	Control	Cy-anide	Control	Cy-anide	Control	Cy-anide
01.8	33.2	10.5	48.8	24.7	100.3	88.0	34.0	2.4	7.9	113.5	35.7	0	28.7	15.9	17.0	18.8	44.7
56.0	39.8	10.0	05.2	33.2	100.5	82.0	55.0	2.4	7.0	02.6	50.7	17.1	53.9	11.7	35.6	10.2	59.4
64.0	43.4	10.1	20.0	25.2	145.0	94.2	77.6	6.7	9.0	06.1	50.0	32.5	42.2	12.0	19.0	16.7	23.1
05.4	47.6	14.0	23.6	20.9	09.2	90.2	75.2	3.2	10.2	02.2	51.0	30.6	07.2	8.0	4.0	18.4	25.0
78.0	43.7	16.3	78.1	39.5	131.0	74.8	38.4	3.2	5.4	82.0	4.9	17.5	130.0	11.6	20.9	17.0	62.4
01.7	38.1	13.8	34.8	20.7	150.0	73.0	43.5	6.3	8.0	04.8	4.6	42.9	72.2	3.2	17.0	15.6	32.2
68.8	22.6	11.8	24.5	28.7	133.0	85.0	44.0	2.8	0.3	64.5	39.3	59.8	07.0	10.1	9.4	14.1	33.9
68.7	32.8	17.7	31.1	74.2	74.2	74.0	4.7	3.1	07.0	32.1	58.0	75.5	4.0	11.5	10.7	33.5	—
Average...05.5	30.8	14.9	40.7	28.0	118.0	55.3	4.0	7.5	87.9	35.4	33.0	67.1	9.7	17.0	10.3	39.2	—
t* .....	7.0	8.3	8.2	3.0	3.3	5.6	2.9	0.01	0.01	0.01	0.00	4.3	—	—	—	—	—
P† .....	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

\* Difference between the means divided by the standard error of the difference of the means.  
 † Probability value.



phate and phosphocreatine, show a marked and significant decrease. Adenosine diphosphate, as might be expected, increases as the adenosine triphosphate content of the tissue is lowered. Increases in the concentration of inorganic phosphate were also very marked.

#### DISCUSSION

The present work shows that inactivation of cytochrome oxidase by cyanide, previously demonstrated *in vitro*, may also be demonstrated to exist *in vivo*. It would appear that, in the intact animal tissue, anoxia, whether brought about by arterial undersaturation (9), by inadequate peripheral circulation,<sup>1</sup> or by inactivation of cytochrome oxidase, results in a shift from aerobic to anaerobic metabolism and a depletion of high energy phosphorus compounds.

The question arises, however, whether there may not be differences in the degree of these changes in different tissues in the various types of anoxia. LePage<sup>1</sup> found that the concentration of lactic acid was higher by 20 to 75 per cent in moribund shocked rats than in rats killed by asphyxiation and incubated for 20 minutes. In non-moribund, shocked rats the decrease in concentration of the high energy phosphorus compounds and the increases in concentration of inorganic phosphate and lactic acid were much greater in the liver than in the brain. Comparisons of our results with those of LePage<sup>1</sup> reveal that the decrease in the concentration of phosphocreatine and the increases in lactic acid and inorganic phosphate were much more marked in the brain of the cyanide-poisoned rat than in that of the non-moribund, shocked rat.

Further experiments are indicated, however, to define more precisely the pattern of chemical changes in tissue in anoxia due to cytochrome oxidase inactivation ("histotoxic" anoxia). Cyanide poisoning involves disturbances in respiration which may contribute an element of "anoxic" anoxia, and at certain stages may affect cardiac function<sup>3</sup>, and thus lead to inadequate peripheral circulation and an element of "stagnant" anoxia. As has been noted, in stagnant anoxia due to shock, the extent and pattern of chemical changes in tissue vary with the particular tissue.<sup>1</sup> There are no data available on the pattern of chemical changes in tissue in anoxic anoxia. The pattern of chemical changes in different tissues in cyanide poisoning, the extent to which these are complicated by incidental elements of anoxic and stagnant anoxia, and the possible relation of these changes in the various tissues to the degree of cytochrome oxidase inactivation remain to be studied.

#### SUMMARY

Rats, injected intraperitoneally with 5 mg. of NaCN per kilo, showed approximately a 50 per cent decrease in cytochrome oxidase activity in

<sup>1</sup> Wexler, J., and Tatum, H. J.. personal communication.

the brain. The brains of these cyanide-poisoned rats showed significant decreases in the concentrations of glycogen, phosphocreatine, and adenosine triphosphate, and significant increases in the concentrations of inorganic phosphate, lactic acid, hexose diphosphate, phosphoglycerate, and phosphopyruvate.

These results indicate that anoxia in tissue induced by inactivation of cytochrome oxidase results in a shift from aerobic to anaerobic metabolism and a depletion of high energy phosphorus compounds.

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# BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

## III. A TRANSFORMATION INVOLVING PHENYLALANINE

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The metabolite function and biogenesis of nutrillites have received increased attention in recent years with the development of new techniques, particularly in the field of biochemical genetics (1). A recently developed technique which may be termed *inhibition analysis* has been successful in readily determining the biosynthetic sequence of metabolites (2, 3).

In expanding this method of determining the metabolic interrelationship of cell constituents, analogues which would competitively inhibit the functioning of phenylalanine were considered for the purpose of studying some transformations in which this amino acid is involved.

Du Vigneaud *et al.* (4) have shown that  $\beta$ -2-thienylalanine inhibits the normal growth of yeast and that the growth inhibition is prevented by phenylalanine. Although Dakin (5) studied the metabolism of  $\beta$ -hydroxyphenylalanine (phenylserine) and did not report any ill effects resulting from its administration to cats, the structure of this substance made it seem to be a likely inhibitor of phenylalanine utilization.

The present paper reports the results of studies of the competitive inhibition of phenylalanine utilization in *Escherichia coli* with  $\beta$ -hydroxyphenylalanine and  $\beta$ -2-thienylalanine and the effects of phenylalanine and tryptophane on this inhibition. These results indicate that tryptophane may be a precursor of phenylalanine.  $\beta$ -Hydroxyphenylalanine also inhibits competitively the functioning of phenylalanine in *Lactobacillus arabinosus* 17-5 and in *Streptococcus faecalis* R, both of which require an outside source of phenylalanine for maximum rate of growth.

### EXPERIMENTAL

*dl*- $\beta$ -Hydroxyphenylalanine—One of the two stereoisomeric forms of *dl*- $\beta$ -hydroxyphenylalanine was prepared by the method of Erlenmeyer and Früstück (6).

*dl*- $\beta$ -2-Thienylalanine—*dl*- $\beta$ -2-Thienylalanine (100 mg.) was supplied through the courtesy of Dr. Vincent du Vigneaud (4).

*Phenylpyruvic Acid*—Phenylpyruvic acid was prepared by the method

of Hemmerle (7) by the condensation of phenylacetonitrile and diethyl oxalate and hydrolysis of the resulting cyano ester. A second preparation of phenylacetic acid was made by treating benzylmagnesium chloride with dry ice (8), esterifying the acid with ethyl alcohol, condensing the ester with diethyl oxalate, and hydrolyzing the product (9).

*Tryptophane*—The *dl*-tryptophane was obtained from Merck and Company, Inc., and assayed for phenylalanine by the method of Stokes *et al.* (10). Varying amounts up to 4 mg. of *dl*-tryptophane showed no activity in replacing phenylalanine in the microbiological assay. The sample, therefore, could not have contained any appreciable amount of phenylalanine.

*Testing Methods*—The basal medium used in tests with *Escherichia coli* has been previously described (2). The casein hydrolyzed with trypsin was omitted. The basal medium used for *Lactobacillus arabinosus* 17-5 was essentially that described by McMahan and Snell (11), which contained no phenylalanine. A basal medium described by Stokes *et al.* (10), which contained no phenylalanine, was used for *Streptococcus faecalis* R. Time and temperature of incubation are given in Tables I to IV. The *Escherichia coli* culture was carried by daily transfers in a previously described medium (2) without hydrolyzed casein and supplemented with 10 mg. of *dl*-phenylalanine per 10 cc.

*Results*—As shown in Table I,  $\beta$ -hydroxyphenylalanine inhibits the growth of *Escherichia coli*, and phenylalanine completely prevents the inhibition below a level of 30 mg. of inhibitor per 10 cc., at which point  $\beta$ -hydroxyphenylalanine becomes irreversibly toxic; that is, its toxicity is not prevented by phenylalanine. The ratio of  $\beta$ -hydroxyphenylalanine to phenylalanine at which complete inhibition of growth results (the antibacterial index) (12) is approximately 1000. As judged from the antibacterial index and from the amount of inhibitor necessary to prevent growth in the absence of added phenylalanine, it appears that this strain of *Escherichia coli* is able to synthesize an amount of phenylalanine equivalent to a concentration in the medium of approximately 0.3 to 1  $\gamma$  per 10 cc.

The effect of tryptophane on the toxicity of  $\beta$ -hydroxyphenylalanine is shown in Table II. Tryptophane is approximately one-tenth as effective as phenylalanine at low concentrations in preventing the toxicity of the inhibitor but, as compared to phenylalanine, becomes progressively less effective at higher levels.

The effect of 200  $\gamma$  of *dl*-tryptophane on the antibacterial index is shown in Table I. The influence of the tryptophane becomes negligible at high levels of phenylalanine, and is not that of changing the antibacterial index.

The inhibition of growth of *Escherichia coli* by  $\beta$ -2-thienylalanine and its prevention by phenylalanine and tryptophane are shown in Table III.

In this case, the ratio of inhibitor to phenylalanine at which complete inhibition of growth results is approximately 100; hence,  $\beta$ -2-thienylalanine

TABLE I

Prevention of  $\beta$ -Hydroxyphenylalanine (Phenylserine) Toxicity by Phenylalanine and Tryptophane

Test organism, *Escherichia coli*; incubated 16 hours at 37°.

$\beta$ -Hydroxyphenylalanine	dl-Phenylalanine	Galvanometer readings	
		Without added tryptophane	With added dl-tryptophane, 200 $\gamma$ per 10 cc.
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.		
0	0	47.0	47.0
100	0	46.0	46.0
300	0	5.0	45.5
1,000	0	2.0	46.0
3,000	0		44.0
10,000	0		2.0
0	3	42.0	48.0
300	3	43.0	47.3
1,000	3	36.0	49.0
3,000	3	5.0	48.5
10,000	3	2.0	2.0
0	10	46.0	47.2
1,000	10	43.5	48.2
3,000	10	30.0	47.0
10,000	10	7.5	4.0
30,000	10	2.0	2.0
0	30	43.0	47.0
3,000	30	41.5	48.0
10,000	30	26.2	32.0
30,000	30	2.0	2.0
100,000	30	2.0	2.0
0	100	46.5	45.9
10,000	100	45.0	46.0
30,000	100	15.5	12.5
100,000	100	2.0	2.0
30,000	300	10.2	
30,000	1,000	16.0	
30,000	3,000	22.0	
30,000	10,000	20.0	
Antibacterial index.....		1000 Ca.	1000 Ca.

is approximately 10 times as effective in inhibiting the growth of *Escherichia coli* as is  $\beta$ -hydroxyphenylalanine. Tryptophane is about one-tenth as effective as phenylalanine in preventing the inhibition of growth at low

concentrations of inhibitor but, compared with phenylalanine, becomes progressively less effective at higher levels of thienylalanine.

TABLE II  
Effect of Tryptophane on Toxicity of  $\beta$ -Hydroxyphenylalanine  
Test organism, *Escherichia coli*; incubated 15 hours at 39°.

$\beta$ -Hydroxyphenylalanine $\gamma$ per 10 cc.	Galvanometer readings	
	<i>l</i> -Tryptophane, 0 $\gamma$ per 10 cc.	<i>dl</i> -Tryptophane
0	57.5	
100	48.5	
300	6.5	
1,000	3.0	
	3 $\gamma$ per 10 cc.	6 $\gamma$ per 10 cc.
0	57.0	58.0
100	54.0	55.0
300	25.5	43.0
1,000	3.5	4.5
	10 $\gamma$ per 10 cc.	20 $\gamma$ per 10 cc.
0	56.5	56.0
300	54.0	54.0
1,000	7.0	3.5
3,000	3.0	4.0
	30 $\gamma$ per 10 cc.	60 $\gamma$ per 10 cc.
0	56.5	56.5
300	55.0	55.0
1,000	24.5	19.5
3,000	4.0	3.5
	100 $\gamma$ per 10 cc.	200 $\gamma$ per 10 cc.
0	56.5	57.5
1,000	55.0	55.0
3,000	11.0	8.5
10,000	3.0	7.0
	1000 $\gamma$ per 10 cc.	2000 $\gamma$ per 10 cc.
0	58.0	56.0
3,000	57.5	54.0
10,000	3.0	4.0

Growth of both *Lactobacillus arabinosus* 17-5 and *Streptococcus faecalis* R is inhibited by  $\beta$ -hydroxyphenylalanine, as shown in Table IV. The

TABLE III

Prevention of  $\beta$ -2-Thienylalanine Toxicity by *dl*-Phenylalanine and *dl*-Tryptophane  
 Test organism, *Escherichia coli*; incubated 16 hours at 37°.

<i>dl</i> - $\beta$ -2-Thienylalanine  $\gamma$ per 10 cc.	Galvanometer readings	
	<i>dl</i> -Phenylalanine, 0 $\gamma$ per 10 cc.	<i>dl</i> -Tryptophane
0	43.5	
3	28.5	
10	7.0	
30	5.0	
	10 $\gamma$ per 10 cc.	20 $\gamma$ per 10 cc.
0	43.0	45.0
10	43.0	41.0
30	34.0	39.0
100	29.0	4.0
300	26.0	3.0
1000	5.0	4.0
	30 $\gamma$ per 10 cc.	60 $\gamma$ per 10 cc.
0	44.0	46.0
30	44.0	41.0
100	43.0	36.0
300	40.0	4.5
1000	33.0	3.5
3000	6.0	
	100 $\gamma$ per 10 cc.	200 $\gamma$ per 10 cc.
0	45.0	43.0
100	43.0	43.0
300	42.0	17.0
1000	38.5	4.0
3000	35.0	4.0
	30 $\gamma$ per 10 cc.	600 $\gamma$ per 10 cc.
0	47.0*	44.0
100	45.0*	45.0
300	42.0*	43.0
1000	36.5*	5.0
3000	6.0*	
		2000 $\gamma$ per 10 cc.
0		43.0
300		43.5
1000		31.0
3000		5.0
Antibacterial index	100 Ca.	

\* Contains 200  $\gamma$  of *dl*-tryptophane per 10 cc.



inhibitory action of the compound is prevented by sufficient amounts of phenylalanine below levels of 30 to 100 mg. per 10 cc., at which point the toxicity of  $\beta$ -hydroxyphenylalanine is not completely prevented by any amount of phenylalanine. The competition of  $\beta$ -hydroxyphenylalanine and phenylalanine results in an antibacterial index of approximately 200 for both *Lactobacillus arabinosus* 17-5 and *Streptococcus faecalis* R. In

TABLE IV

Prevention of  $\beta$ -Hydroxyphenylalanine (Phenylserine) Toxicity by dl-Phenylalanine  
Test organisms incubated 16 hrs. at 30°.

$\beta$ -Hydroxyphenylalanine	dl-Phenylalanine	Galvanometer readings	
		<i>Lactobacillus arabinosus</i>	<i>Streptococcus faecalis</i> R
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.		
0	0	12.5	1.0
0	50	44.0	47.0
300	50	38.5	37.0
1,000	50	23.0	33.0
3,000	50	11.0	15.0
10,000	50	5.0	3.0
0	100	56.5	53.0
300	100	42.0	52.0
1,000	100	36.0	47.0
3,000	100	34.0	34.0
10,000	100	9.0	5.0
0	300	54.0	55.0
1,000	300	50.0	58.0
3,000	300	35.0	54.0
10,000	300	45.0	54.0
30,000	300	10.0	17.0
100,000	300	5.0	4.0
0	1,000	56.0	58.0
100,000	1,000	10.0	23.0
100,000	3,000	11.5	33.0
100,000	10,000	14.0	35.0
100,000	30,000	23.0	35.0
Antibacterial index		200 Ca.	200 Ca.

contrast to *Escherichia coli*, which synthesizes phenylalanine, these organisms, under the conditions of testing, require added phenylalanine for maximum rate of growth.

The effect of other amino acids on the inhibition of the growth of *Escherichia coli* by  $\beta$ -hydroxyphenylalanine indicates that some of these may be related metabolically to phenylalanine. None of the amino acids were as

effective as tryptophane and phenylalanine; however, the activity of those amino acids which may be considered substituted alanines in preventing the toxicity of  $\beta$ -hydroxyphenylalanine is sufficient to warrant further investigation.

In an attempt to determine how tryptophane and phenylalanine were metabolically interrelated,  $\beta$ -phenylethylamine, phenylsulfuric acid, and phenylpyruvic acid were tested and found to show no effect on the toxicity of thienylalanine or  $\beta$ -hydroxyphenylalanine either in the presence of or in the absence of tryptophane. A sample of phenylpyruvic acid prepared by the acid hydrolysis of ethyl  $\beta$ -cyanophenyl pyruvate was about one-thirtieth as active as phenylalanine, whereas phenylpyruvic acid prepared by an alternate method was inactive. The formation of ammonia during the hydrolysis of the cyano compound presumably produced a small amount of phenylalanine, which caused the preparation to show slight activity.

#### DISCUSSION

Competitive inhibition studies, of the type illustrated by these data, may be conveniently interpreted by assuming that the inhibition of growth of a microorganism results from the competition of the analogue with a metabolite for a specific enzyme, that the analogue-enzyme complex is incapable of performing the normal function of the metabolite, and that the growth of the microorganism is a function of some product normally formed by the blocked enzyme, when this system becomes a limiting factor of growth (2). According to this concept,  $\beta$ -hydroxyphenylalanine and  $\beta$ -2-thienylalanine block the utilization of phenylalanine in a process essential to the growth of the organism.

Substances other than the metabolite which reverse the toxicity of an analogue may generally act in two ways; they may be a precursor of the metabolite, in which case they will form more metabolite and competitively reverse the toxicity, or they may be a product of the blocked enzyme system, in which instance amounts sufficient for growth will completely prevent the inhibition of growth caused by the "blocked" enzyme system. If another enzyme system is involved in the utilization of the metabolite, a new antibacterial index applying to this new enzyme system may be obtained in case the inhibitor blocks such a function.

Since tryptophane was highly effective in reversing the toxicity of phenylalanine analogues, it must be considered from both of these standpoints. If tryptophane were a product of the blocked enzyme system, two types of changes might result: (a) the inhibitor would no longer be effective (over the range of concentration in which the inhibitor is competitive with phenylalanine) when the single blocked enzyme system is the only one

capable of being blocked by the inhibitor, or (b) a higher antibacterial index would be obtained when other enzyme systems can be blocked by the inhibitor. The antibacterial index determined in the presence of relatively large amounts of phenylalanine showed no appreciable change in the presence of tryptophane. The effect of tryptophane is thus "diluted" by large amounts of phenylalanine. This is true for inhibition of *Escherichia coli* with either thienylalanine or hydroxyphenylalanine. Thus, the addition of 200  $\gamma$  of *dl*-tryptophane per 10 cc. appears to result in the synthesis of approximately 10  $\gamma$  of phenylalanine (based on *dl*). Hence, the ability of *Escherichia coli* to utilize tryptophane in forming additional phenylalanine would account for the prevention of toxicity of phenylalanine analogues by tryptophane. The fact that tryptophane becomes relatively less effective at higher levels also indicates that the effect is that of a precursor rather than a product which normally is ineffective above levels required for growth.

The transformation suggested above does not necessarily imply that any gross part of the tryptophane molecule is converted to phenylalanine. The actual mechanism of the reaction is at present obscure, but three other possibilities immediately present themselves: transcarboxylation of tryptophane with  $\beta$ -phenylethylamine, transamination with phenylpyruvic acid, and "transalanination" with phenylsulfuric acid. Further study is being given this problem.

The reversing effects of the other amino acids, particularly substituted alanines, also merit further study.

The authors are indebted to Joanne Macow Ravel and Marjorie Rogers Korn for their assistance in some of the testing.

#### SUMMARY

$\beta$ -Hydroxyphenylalanine inhibits the growth of *Escherichia coli*, *Lactobacillus arabinosus* 17-5, and *Streptococcus faecalis* R. The inhibition is prevented competitively by phenylalanine. The antibacterial indices are approximately 1000, 200, and 200, respectively. At a concentration of 30 to 100 mg. per 10 cc.,  $\beta$ -hydroxyphenylalanine becomes irreversibly toxic with respect to reversal with phenylalanine.

Thienylalanine also inhibits the growth of *Escherichia coli*, and the inhibition is prevented by sufficient amounts of phenylalanine. The antibacterial index is approximately 100.

Tryptophane prevents the toxicity of both  $\beta$ -hydroxyphenylalanine and thienylalanine over rather wide ranges of concentration. Following a method termed inhibition analysis, the effect of tryptophane was characteristic of a precursor. It is suggested that *Escherichia coli* has the

ability to transform added tryptophane into phenylalanine. The exact nature of the transformation is being investigated.

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# THE PURIFICATION OF TOXIN FROM CLOSTRIDIUM BOTULINUM TYPE A

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Until recently it could be said that the status of our knowledge of bacterial exotoxins had not changed since Roux and Yersin in 1888 (1) first obtained the filtrable diphtheria toxin. Indeed, Topley and Wilson in 1936 (2) stated, "We have not yet succeeded in isolating any bacterial exotoxin in a chemically pure state, although a considerable degree of concentration has been attained by various methods of fractional precipitation." The same year Eaton (3) brought to light the first proof that a bacterial toxin (diphtheria) was a single protein-like substance by reporting the isolation of the toxin in a purified form. In 1937, Pappenheimer (4) isolated and characterized a toxic protein which appeared to be identical with diphtheria toxin. The properties of this protein were almost identical with those of the substance isolated by Eaton. With the protein nature of one bacterial toxin established, work on other bacterial toxins (streptolysin (5), scarlet fever (6, 7), tetanus (8), and *Clostridium botulinum* type A<sup>1</sup>) has confirmed the validity of the concept that bacterial exotoxins are proteins.

When a toxin has been identified as a protein, proof of its purity presents many complex problems. Kekwick and McFarlane (9) present three criteria of a stable chemical compound: constancy of chemical composition, homogeneity of physical properties, and constancy of solubility. In work with a biologically active protein, the first criterion should of course include constancy of chemical composition and activity. A few of these criteria have been applied to bacterial toxins; e.g., diphtheria toxin (10) and scarlet fever toxin (7).

Previous work by Sommer (11) on *Clostridium botulinum* type A toxin has yielded a preparation containing 250,000,000 mouse m.l.d.<sup>2</sup> per gm. This toxin was prepared in a peptic digest medium by precipitation at pH 3.5 to 4.0 and resolution in sodium acetate buffer. Since washed dried organisms were found to contain 100,000,000 mouse m.l.d. per gm., Sommer (11) assumed that the chemically pure toxin must have a potency many times greater.

<sup>1</sup> Lamanna, C., McElroy, O. E., and Eklund, H. W., *Science*, in press.

<sup>2</sup> This term is not defined by Sommer; in this paper, 1 m.l.d. is defined as the smallest amount of toxin which causes death within 4 days of all mice injected.

In the present study,<sup>3</sup> Sommer's method of purification by acid precipitation and acetate buffer resolution has been extended according to the usual methods of alcohol and salt fractionation of proteins. The steps of fractionation have been followed by mouse m.l.d. titrations and nitrogen determinations and also by ultraviolet absorption spectra and electrophoresis. The fractionations, to be described in detail below, have resulted in preparations which are electrophoretically homogeneous, show characteristic protein properties, and have a maximum value of  $220 \times 10^6$  mouse m.l.d. per mg. of nitrogen. Crystalline material has been obtained from these preparations.<sup>4</sup> The variation of electrophoretic mobility with pH has been studied. Other physicochemical properties of the purified toxin are being studied and will be reported in a subsequent paper.

### Methods

*Strains*—The Hall strain of *Clostridium botulinum* type A was used in preparing all cultures for toxin production. The strain was maintained in 1 per cent peptone, beef heart infusion broth, which contained chopped beef heart in the bottom of each tube. After an initial growth period of 24 hours at 34°, the cultures were stored at 4° until needed. In the preparation of seed, the culture was grown 18 to 24 hours in chopped beef heart medium, then transferred to a 2 per cent pepticase medium for the same length of time. 2 ml. of the seed culture were added to each 100 ml. of medium, which was planted for toxin production.

*Medium*—The medium<sup>5</sup> used for toxin production was prepared as follows: 2 per cent pepticase (a commercial tryptic digest of casein); 0.75 per cent corn steep liquor (containing approximately 60 mg. of N per ml.); tap water to volume; pH adjusted to 7.5; and 16 liters dispensed into each 5 gallon Pyrex bottle.

For more uniform toxin production the pepticase was treated with charcoal before being added to the medium. To each 100 gm. of pepticase, tap water was added to bring the volume to 500 ml. Solution was obtained by heating the mixture in the autoclave at 15 pounds pressure for 5 minutes. As soon as the pepticase solution was removed from the autoclave, 5 gm. of U. S. P. charcoal were added and the mixture was shaken intermittently

<sup>3</sup> This work was considered to be of such an important nature that two groups working independently were assigned to it. The study reported here was undertaken as preliminary to an investigation into the nature of *Clostridium botulinum* type A toxoid.

<sup>4</sup> Crystalline *Clostridium botulinum* type A toxin was previously prepared by Dr. Carl Lamanna and coworkers, working independently at Camp Detrick and using completely different methods.

<sup>5</sup> Pappenheimer, A. M., Jr., Manire, P., and Mueller, J. H., personal communication.

for 30 minutes. The charcoal was then removed by filtration. This amount of pepticase was sufficient for preparing 5000 ml. of medium.

The corn steep liquor was treated with alkali and heated before it was added to the medium. To 100 ml. of crude corn steep liquor were added 200 ml. of cold tap water plus enough NaOH to bring to pH 9.0 to 9.5. Tap water was added to bring the volume to 400 ml. The mixture was heated in the autoclave at 15 pounds pressure for 5 minutes, then clarified by filtration. This filtrate was stored at 4° under toluene and used as needed. 30 ml. of this solution were used to prepare 1000 ml. of medium.

The bottles were autoclaved  $1\frac{1}{2}$  hours at 15 pounds pressure, commencing when the temperature of the exhaust line had reached 115.5°. The autoclave was opened 2 hours after the steam was turned off. The medium was allowed to cool in the autoclave overnight.

0.5 per cent dextrose was added when the medium had cooled to 40° (400 ml. of a sterile 20 per cent solution to 16 liters of medium).

*Growth*—As soon as the medium had cooled to 35°, each bottle was planted with 300 ml. of seed. All cultures were grown 4 days at 33–34°. A daily check of the pH was made and adjustment with 5 N HCl or 5 N NaOH was made as necessary to maintain the reaction at pH 5.7 to 6.0. At the end of the growth period each bottle was checked for purity by examining stained smears, and by subculture in nutrient broth, nutrient agar slants, and nutrient agar shake tubes.

*Toxicity Test*—The number of minimum lethal doses per ml. was determined by injecting intraperitoneally into 20 gm. mice 1 ml. volumes of toxin diluted in 0.2 per cent gelatin buffer (pH 6.8).<sup>2</sup>

*Flocculation Tests (Lf)*—Flocculation tests were carried out in order to determine the amount of antitoxin with which the toxin combined *in vitro*. The Lf value of a toxin is the number of units of antitoxin with which 1 ml. of toxin unites and flocculates in the shortest interval of time (12). All Lf determinations were carried out in a water bath at 42° with 1 ml. portions of diluted toxin and amounts of antitoxin<sup>6</sup> (150 units per ml.) varying from 0.03 to 0.20 ml.

*Chemical Determinations*—Total nitrogen was determined by the micro-Kjeldahl method. Total phosphorus was determined by King's (13) method.

*Electrophoresis*—Electrophoresis investigations were carried out in the Tiselius apparatus (14), as modified by Longworth (15). Toxin preparations were dialyzed statically at 2–5° against approximately 10 volumes of buffer, with change of buffer three times daily for 3 days, with measurements of conductivity to determine completion of dialysis (16). Relative concentrations were estimated by tracing the curves obtained under a

<sup>6</sup> Globulin-modified antitoxin, Lederle.



photographic enlarger and determining areas under the traced pattern with a polar planimeter, the same specific refractive increment for all components having been assumed.

*Ultraviolet Absorption Spectra*<sup>7</sup>—Determinations of the ultraviolet absorption spectra were made with the Beckman spectrophotometer with quartz cells of 1 cm. width.

#### EXPERIMENTAL

*Preliminary Study of Nature of Toxin; Stability of Toxin*—Experiments were carried out to confirm the reported inactivation of *Clostridium botulinum* type A toxin by alkali and heat (17). It was found that at room temperature the toxin was most stable between pH 1.0 and 6.0 with maximum stability between 4.0 and 5.0, while above pH 7.0 the toxin was rapidly destroyed. It was found also that a temperature of 60° at pH 5.0 was sufficient to destroy 100,000 m.l.d. in 5 minutes. In order to prevent inactivation of the toxin during the purification procedure, the pH was maintained below 7.0 by means of the buffers (1 per cent sodium acetate and 1 per cent potassium phosphate) and the temperature was maintained at 4° except during precipitation with Na<sub>2</sub>SO<sub>4</sub>, which was carried out at room temperature.

*Fractionation with Acid, Alcohol, and Na<sub>2</sub>SO<sub>4</sub>*—The whole culture containing both toxin and bacteria was adjusted to pH 3.5 and allowed to stand at room temperature for several days. The precipitate which formed was separated and washed by decantation several times with distilled water. The toxin was extracted by suspending the precipitate in one-fourth the original volume of 1 per cent sodium acetate solution adjusted to pH 6.5. After three such extractions the supernatants were combined and the insoluble residue<sup>8</sup> was discarded. The extracts were adjusted to pH 3.5. The precipitated toxin was separated, washed twice with distilled water, and then redissolved in one-fourth the original volume of 1 per cent sodium acetate solution at pH 6.5. This concentrated toxin solution (Fraction A, Table I) was used for alcohol fractionation.

Fractionation with ethyl alcohol was carried out at 4° at pH 6.5. Precipitates were centrifuged and washed with the same concentration of alcohol (buffered at pH 6.5) used in precipitation, and then taken up in distilled water. Fractions were obtained with 10, 20, 40, and 50 per cent ethanol. Table I summarizes the data obtained for each fraction.

Fraction II, obtained with 20 per cent alcohol, was fractionated further with Na<sub>2</sub>SO<sub>4</sub> at pH 6.5 at room temperature. The toxin was first precipitated by adding a half volume of a saturated Na<sub>2</sub>SO<sub>4</sub> solution prepared

<sup>7</sup> These determinations were carried out by Dr. E. C. Smith.

<sup>8</sup> This residue may carry with it as much as 50 per cent of the toxin.

at 33°. The precipitate was removed by centrifuging and dissolved in distilled water. This toxin was then treated with  $\text{Na}_2\text{SO}_4$  solution to yield two fractions, one precipitated at 0.18 saturation, the other at 0.4 saturation (Fraction II-S-2). The data on these fractions are also given in Table I.

TABLE I

Results of Preliminary Fractionation of Crude *Clostridium botulinum* Type A Toxin

Product	Volume	Total N	N:P ratio	Mouse m.l.d. in millions			Electrophoretic data at pH 6.52-6.82, relative concentration		
				Per ml.	Total	Per mg. N	Component A, toxin	Component B, colored	Component C, nucleic acid
	ml.	mg. per ml.					per cent	per cent	per cent
Whole culture. ....	44,000	2.18*		0.8	35,000	0.37*			
Fraction A, concentrated toxin solution. ....	295	1.37		8	2,400†	6.0			
Fraction I, 10% alcohol. ....	100	0.743	16.2	8	800	11.0			
" II, 20% " ....	105	0.904	13.2	32	3,400†	35.0	33	58	9
" III, 40% " ....	65	0.288	5.1	2	130	7.0			
" IV, 50% " ....	90	1.25	2.1	<0.5	<45	<0.4			
" II-S-1, 0.18 saturated $\text{Na}_2\text{SO}_4$ . ....	40	0.740	15.0	4	160	5.0			
Fraction II-S-2, 0.40 saturated $\text{Na}_2\text{SO}_4$ . ....	25	0.320	14.4	32	800	100.0	63	37	0
Fraction II-S-2', pH 5.0 supernatant. ....	15						68	32	0
Fraction 287-IV-A,‡ water-insoluble. ....	15	0.300				60	100	0	0

\* Value obtained from another experiment.

† The discrepancy in these values may be due to inaccuracies in mouse m.l.d. titrations in which 2-fold dilutions are used.

‡ Prepared from a different lot of crude toxin. The unusually low m.l.d. per mg. of N is explained in the text.

It was possible by further fractionation to obtain a greater degree of purity of Fraction II-S-2. By adjustment to pH 5.0, a considerable amount of colored impurity precipitated out at 6°. This material was removed by centrifugation. The toxin was precipitated in 0.4 saturated  $\text{Na}_2\text{SO}_4$  at pH 6.5 and redissolved in cacodylate buffer at pH 6.78 (Fraction II-S-2'). The data on this fraction are shown in Table I.

A new lot of toxin was prepared and purified in the same manner as

described above. One additional step in the fractionation procedure consisted of precipitation of the toxin by dialysis against distilled water. The toxin was separated and redissolved by dialysis against approximately 0.1 per cent NaCl at 4°. By this procedure a small amount of colored

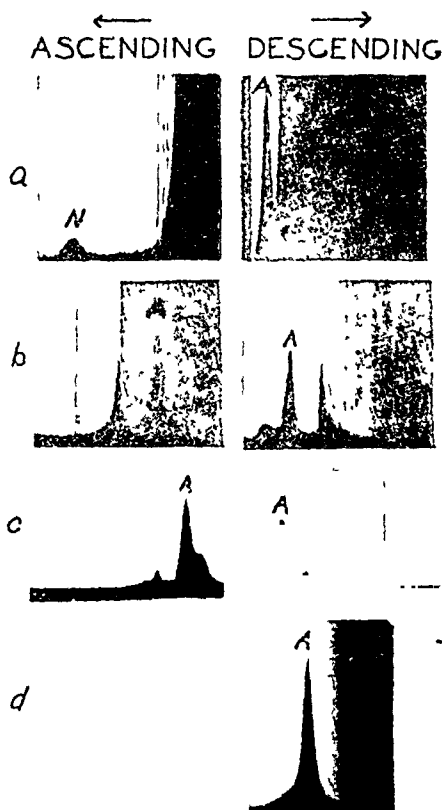


FIG. 1. Electrophoretic patterns of *Clostridium botulinum* toxin fractions showing progressive increase in the relative concentration of Component A. Component N is nucleic acid. The patterns were obtained in buffer of 0.10 ionic strength (0.08 N NaCl, 0.02 N sodium cacodylate, 0.004 N cacodylic acid). (a) Fraction II at pH 6.52 after 8580 seconds at 2.45 volts per cm.; (b) Fraction II-S-2 at pH 6.78 after 15,270 seconds at 3.64 volts per cm.; (c) Fraction II-S-2' at pH 6.78 after 8585 seconds at 3.66 volts per cm.; (d) Fraction 287-IV-A at pH 6.82 after 11,899 seconds at 4.13 volts per cm.

impurity became insoluble and was removed. The supernatant containing the toxin formed a white precipitate in 0.3 saturated  $\text{Na}_2\text{SO}_4$ , which dissolved in cacodylate buffer at pH 6.8 (Fraction 287-IV-A). The data on this fraction are shown in Table I.

The electrophoretic patterns of the four most active fractions above are shown in Fig. 1 and the ultraviolet absorption curves are shown in Fig. 2.

*Results of Preliminary Fractionations*—The data in Table I outline the progress in successive steps in the purification. The column, m.l.d. per mg. of N, gives an indication of the relative purity of each toxin fraction. Thus while the crude culture contained  $0.37 \times 10^6$  mouse m.l.d. per mg. of N, the more purified preparations contained up to  $100 \times 10^6$  mouse m.l.d. per mg. of N.

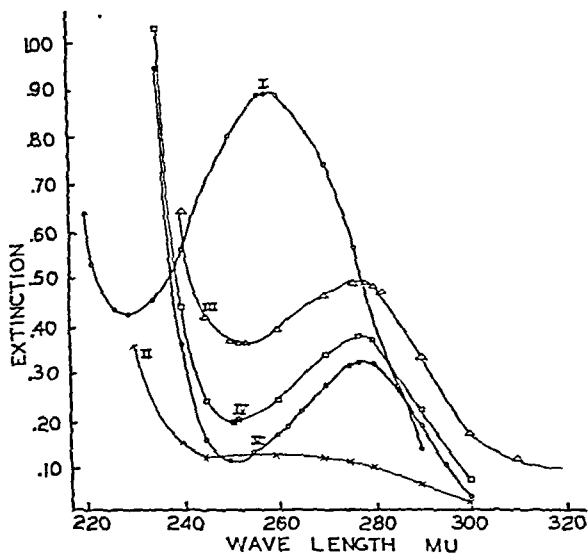


FIG. 2. Ultraviolet absorption spectra of *Clostridium botulinum* nucleic acid and of toxin fractions obtained by alcohol and  $\text{Na}_2\text{SO}_4$  fractionation, and by electrophoretic separation. Curve I, *Clostridium botulinum* nucleic acid; Curve II, Fraction II; Curve III, Fraction II-S-2; Curve IV, Component A from Fraction II; Curve V, Component A from Fraction II-S-2.

By means of fractionation with 20 per cent alcohol a separation was obtained between high phosphorus- and low phosphorus-containing substances. Further fractionation did not further reduce the N:P ratios.

The electrophoretic data, Table I and Fig. 1, illustrate the progressive increase of a single component, with gradual reduction of other components as the fractionation progressed. Although 20 per cent alcohol removed considerable nucleic acid, the electrophoretic pattern showed approximately 9 per cent of a substance with a mobility like that of nucleic acid.

Subsequent fractionation removed all of the fast moving component and finally yielded Fraction 287-IV-A, which was found to contain essentially one component.

By means of electrophoretic separation runs, Component A was isolated free of the other components in both Fraction II and Fraction II-S-2. It was found that in both instances Component A was the toxin.

The low value of  $60 \times 10^6$  mouse m.l.d. per mg. of N of Fraction 287-IV-A, which is essentially homogeneous, is in sharp contrast to the  $100 \times 10^6$  mouse m.l.d. per mg. of N for Fraction II-S-2', which contained only 63 per cent toxin (Component A). One explanation for the low m.l.d. value of Fraction 287-IV-A is a possible conversion of toxin to toxoid.

The ultraviolet absorption curve for Fraction II (Fig. 2) confirms the presence of nucleic acid by a wide absorption band (250 to 280  $m\mu$ ) which is typical of nucleoproteins, or a mixture of nucleic acid and proteins. Fraction II-S-2 and the electrophoretically isolated Component A from both Fraction II and Fraction II-S-2 gave sharp absorption maxima at 278  $m\mu$  with no evidence of nucleic acid absorption.

*Isolation of Nucleic Acid from Culture Fluid*—A nucleic acid was separated from the culture supernatant after precipitation of the crude toxin at pH 3.5. The supernatant was acidified further to pH 2.0 and the precipitate was removed and dissolved at pH 4.0. On addition of an equal volume of 95 per cent alcohol, precipitation took place. The precipitate was dissolved and reprecipitated twice more with 95 per cent alcohol and was finally washed with 95 per cent alcohol and dried. This product contained 13.6 per cent nitrogen and 7.65 per cent phosphorus, and had an N:P ratio of 1.78. The absorption spectrum showed a powerful absorption with a maximum at 258  $m\mu$  (Fig. 2, Curve I).

A strong test for ribose was obtained with Bial's orcinol-HCl reagent. The test for the desoxyribonucleic acid with diphenylamine was only slightly positive. This indicated that the nucleic acid was predominantly the ribose type.

Further evidence for the identity of the isolated nucleic acid with ribonucleic acid was obtained by comparing the absorption spectra of ribonucleic acid, desoxyribonucleic acid, and the *Clostridium botulinum* nucleic acid after reaction with Bial's orcinol-HCl reagent (Fig. 3). The spectrum of *Clostridium botulinum* nucleic acid followed that for ribonucleic acid.

*Isolation and Crystallization of Toxin*—By a study of the preliminary fractionation procedures, it was possible to devise a simplified method for the isolation of the toxin. The procedure is outlined in the accompanying flow diagram.

When the toxin had been sufficiently purified, as shown in the diagram, it was readily obtained in the form of fine needle-like crystals. In order

to get a water-clear solution of toxin before crystallization it was necessary to treat the cloudy, slightly yellow solution with 0.5 saturated ammonium sulfate. By re-solution in phosphate buffer at pH 6.8 and by prolonged centrifugation, it was possible to remove all insoluble material from the concentrated toxin solution. The crystallization was accomplished by dialyzing the concentrated toxin solution containing approximately 1 per cent protein against a low concentration of  $(\text{NH}_4)_2\text{SO}_4$  (0.1 saturated) at pH 6.8 and  $4^\circ$ . The salt concentration was gradually increased until the solution became opalescent; it varied between 0.1 and 0.3 saturation, depending on the concentration of toxin. If precipitation occurred too

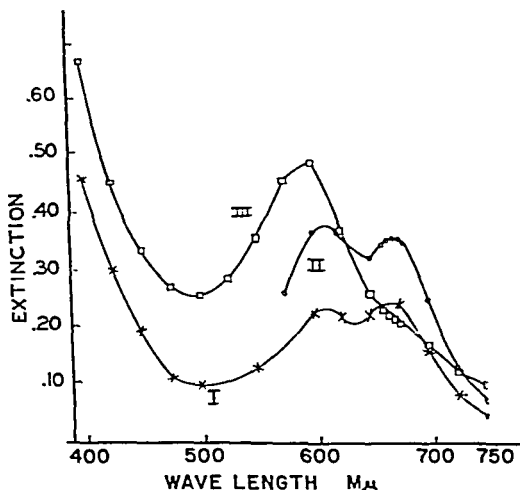


FIG. 3. Absorption spectra of the color obtained after reaction of Bial's orcinol reagent with, Curve I, yeast ribonucleic acid; Curve II, *Clostridium botulinum* nucleic acid; and Curve III, thymus desoxyribonucleic acid.

rapidly, there resulted either minute crystals, an amorphous precipitate, or a mixture of both.

The crystalline toxin had the following properties: (a) The crystals were uniform in size and shape, and free of amorphous material when viewed under the microscope (Fig. 4); (b) the toxin was recrystallized from phosphate buffer, pH 6.8, on dialysis in 0.3 saturated  $(\text{NH}_4)_2\text{SO}_4$  at  $4^\circ$ ; (c) the solution of twice crystallized material had a higher toxicity ( $220 \times 10^6$  m.l.d. per mg. of N) than any previously obtained; (d) the toxin was flocculated by antitoxin with a ratio of 110,000 m.l.d. per Lf, which is slightly higher than the value of 80,000 m.l.d. per Lf found for crude toxin; (e)

*Isolation of Toxin*

*Starting Material*—192 liters crude toxin (whole culture)  $1 \times 10^6$  m.l.d. per ml.; pptd. with acid at pH 3.5; acid ppt. washed 3 times with  $H_2O$  by settling and decantation; acid ppt. in 3 liters adjusted to pH 6.8 with 20%  $K_2HPO_4$  and 5 N NaOH; saturated  $Na_2SO_4$  solution added to 0.4 saturation; supernatant decanted and discarded.

Add water to 5 liters volume; adjust to pH 5.0; let stand 24 hrs.; decant supernatant

Residue I  
Add 1% phosphate buffer, pH 6.8, to 5 liters volume; adjust to pH 6.8; mix well; adjust to pH 5.0; allow to stand; decant supernatant

Supernatant I  
5 liters volume; m.l.d.,  $20 \times 10^6$  per ml.; 52% recovery

Residue II  
Treat same as Residue I

Supernatant II  
5 liters volume; m.l.d.,  $5 \times 10^6$  per ml.; 13% recovery

Residue III  
Discard

Supernatant III  
5 liters volume; m.l.d.,  $1 \times 10^6$  per ml.; 2.6% recovery

Pool Supernatants I, II, and III; adjust to pH 3.5; ppt. forms; centrifuge

Supernatant  
Discard

Ppt.  
Dissolve in 1500 ml. 1% phosphate buffer, pH 6.8; dialyze against  $Na_2SO_4$  to 0.4 saturation; ppt. forms; centrifuge

Supernatant  
Discard

Ppt.  
Dissolve in 500 ml. 1% phosphate buffer, pH 6.8; adjust to pH 4.7; centrifuge

Residue  
Discard

Supernatant  
Dialyze against  $Na_2SO_4$  to 0.3 saturation; ppt. forms; centrifuge

Supernatant  
Discard

Ppt.  
Fraction 303-A-1, dissolve in 130 ml. 1% phosphate buffer, pH 6.8; cloudy, slightly yellow solution; m.l.d.  $500 \times 10^6$  per ml.; 34% recovery; total N, 2.25 mg. per ml.; m.l.d. per mg. N,  $220 \times 10^6$

it was electrophoretically homogeneous (Fig. 5) with a mobility corresponding to that of other preparations of the toxin; (f) the ultraviolet absorption curve of twice crystallized toxin (Fig. 6) showed a single sharp maximum at 278 m $\mu$ .

*Influence of pH on Electrophoretic Mobility*—The influence of pH on the electrophoretic mobility of *Clostridium botulinum* toxin has been investi-



FIG. 4. Photomicrograph of crystals of the toxin produced by *Clostridium botulinum* type A (first crystallization; 450 X).

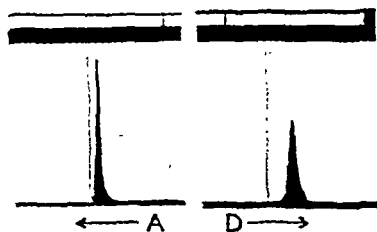


FIG. 5. Electrophoretic pattern of crystalline *Clostridium botulinum* toxin type A in acetate buffer of 0.06 ionic strength at pH 4.12 after 6630 seconds at 5.24 volts per cm.

gated over a wide range of pH values. The results are given in Fig. 7. Precise determination of the isoelectric point was extremely difficult, owing to the very low solubility of the toxin in the neighborhood of the isoelectric point at 1.0°. The protein concentrations ranged from about 0.1 per cent or less (near the isoelectric point) to 0.5 per cent, except for one run at pH 4.47 made with 1 per cent protein. The following uni-univalent buffers were used (18): 0.10  $\mu$  HCl-glycine at pH 3.2; 0.06  $\mu$  sodium acetate-



acetic acid from pH 4.0 to 5.5; 0.10  $\mu$  sodium cacodylate-cacodylic acid from pH 6.0 to 7.0; and 0.06  $\mu$  cacodylate buffer at pH 7.02. The mobility

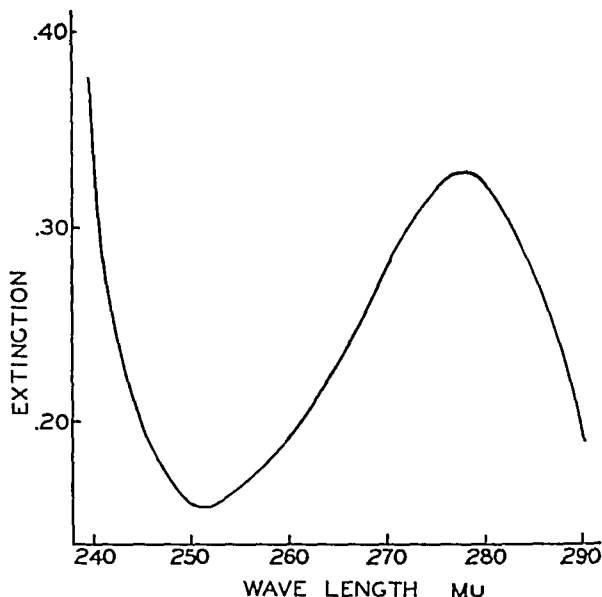


FIG. 6. Ultraviolet absorption spectrum of crystalline *Clostridium botulinum* type A toxin. Concentration, 0.196 mg. per ml.

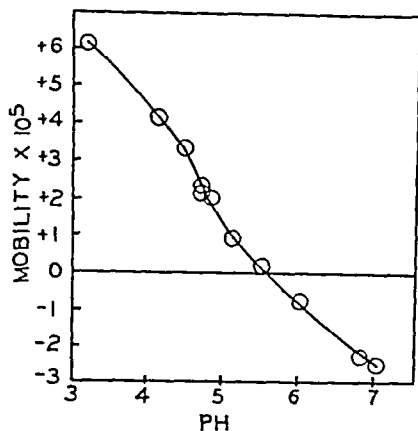


FIG. 7. Mobility of *Clostridium botulinum* type A toxin

determinations were made on electrophoretically homogeneous or nearly homogeneous samples from three different preparations of toxin, with the

exception of two determinations made on a 68 per cent homogeneous preparation. Measurements of pH were made at room temperature with a Beckman glass electrode and pH meter, calibrated with 0.05 M potassium acid phthalate at pH 4.00. The correction of the pH values of the buffers to 1.0° was assumed to be negligible. Mobilities were determined from the rate of movement of the maximum ordinate, which in the case of the purified toxin preparations involved negligible error, because the refractive gradient curves were quite symmetrical (18).

The isoelectric point is estimated from our data to be at pH 5.6, with  $(du/dpH)^{\circ} = 1.9$  near the isoelectric point (19).

The properties of the purified toxin are listed in Table II.

TABLE II  
*Properties\* of Purified Toxin*

Activity  Chemical and physical properties	Toxicity per mg. nitrogen	220 × 10 <sup>6</sup> mouse m.l.d.
	Mg. nitrogen per Lf unit	0.00048
	Nitrogen†	14.1%
	Phosphorus†	0.1%
	Carbohydrate†	Trace
	Biuret test‡	Positive
	Heat coagulability‡	"
	Isoelectric point	pH 5.60
	Absorption maximum extinction	278 mμ
		0.1%; E = 1.67 at 278 mμ
	Insoluble in distilled water	
	Soluble in 0.9% NaCl at pH 5.6	
	Pptd. by 0.4 saturated Na <sub>2</sub> SO <sub>4</sub>	
	" " 0.5 " (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	

\* Determined on twice crystallized toxin unless otherwise indicated.

† Determined on a lyophilized sample of 90 per cent electrophoretically homogeneous toxin.

‡ Determined on toxin crystallized once.

# DISCUSSION

One of the difficult problems in the isolation of a bacterial toxin is that of handling large volumes of fluid and recovering small amounts of active material. This problem was present in the purification of *Clostridium botulinum* toxin, since each liter of whole culture contained only about 20 mg. of toxin. By utilizing the acid precipitation technique at pH 3.5 it was possible to recover a portion of the toxin in a greatly purified state in a reduced volume. The data in Table I show that a 20-fold increase in purity of the toxin was attained by this one step, although only about 7 per cent of the toxin was recovered. Later work in which the toxin was extracted promptly from the acid precipitate showed recoveries approaching 70 per cent of the original toxicity of the whole culture.

The preliminary fractionation experiments with alcohol and  $\text{Na}_2\text{SO}_4$  yielded information as to the nature of the impurities in the crude toxin (Table I). By means of 20 per cent alcohol it was possible to separate a highly active, low phosphorus-containing fraction ( $\text{N:P} = 13.2$ ) from fractions containing more phosphorus ( $\text{N:P} = 5.05$  and  $2.08$ ) and with much lower activity, thus showing phosphorus-containing impurities to be present. Much of this phosphorus-containing impurity was nucleic acid, since actual isolation from the culture fluid, ultraviolet absorption, and electrophoretic patterns showed it to be present in the crude toxin, whereas purified preparations showed no evidence of it. The ultraviolet absorption studies (Fig. 2) showed the transformation from nucleoprotein absorption (250 to  $280 \text{ m}\mu$ ) of the crude toxin to protein absorption alone at about  $280 \text{ m}\mu$ , as the purification proceeded.

The presence also of a non-nucleic acid, phosphorus-containing impurity was indicated by an examination of the data on Fractions II and II-S-2. Although fractionation of Fraction II with  $\text{Na}_2\text{SO}_4$  to give Fraction II-S-2 caused practically no change in the  $\text{N:P}$  ratio, the electrophoretic patterns showed a complete disappearance of the nucleic acid component (Fig. 1), and ultraviolet absorption data showed a sharp decrease in nucleic acid absorption (Fig. 2, Curves II and III).

The fractionation procedures which are described in the early parts of the experimental sections, and which were followed closely by ultraviolet absorption, electrophoresis, and toxicity, are presented to show how the conditions necessary for the best method of purification were developed. The effects of repeated fractionation with alcohol were not studied further. The selection of  $\text{Na}_2\text{SO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$  as fractionating agents does not imply that alcohol would not work just as well.

Maximum toxicity per mg. of nitrogen, electrophoretic homogeneity, and a single ultraviolet absorption band at  $278 \text{ m}\mu$  have been used as criteria for following the various purification procedures. One fraction which fulfilled the latter two criteria had a toxicity of only  $60 \times 10^6$  mouse m.l.d. per mg. of nitrogen, which was much lower than the maximum value of  $220 \times 10^6$  mouse m.l.d. per mg. of nitrogen. On examination of the flocculating power of this toxin with antitoxin, it was found that 30,000 mouse m.l.d. constituted 1 Lf unit of toxin, as compared with 80,000 m.l.d. per Lf unit which was obtained with fresh toxin. One explanation of the reduction in toxicity of this fraction is a possible spontaneous conversion of toxin to toxoid. The validity of this explanation depends upon the assumption (1) that the flocculating power of a toxin is not affected by this conversion process, (2) that the proportion of toxin to toxoid in crude toxin is fairly constant from one preparation to another, and (3) that toxoid has the same electrokinetic properties as toxin. In regard to the first two

assumptions, no evidence has been found to invalidate them. As to the third assumption, nothing is known regarding the properties of the spontaneously formed toxoid.

The final method of isolation of the toxin shown in the flow diagram was found to give a 34 per cent recovery of toxin. This procedure is a refinement and combination of procedures developed in the preliminary phase of this work. It was found to be very important to carry out the  $\text{Na}_2\text{SO}_4$

TABLE III

*Comparison of Some Properties of Biologically Active Bacterial Proteins*

Toxin	Activity per mg.	Activity per Lf	N	S	Iso-electric point	Biuret test	
			$\gamma$ N per Lf	per cent	per cent		
Diphtheria (4)	10,000 guinea pig m.l.d.	30 m.l.d.	0.46	16	0.75	4.1	Positive
" (3)	10,000 guinea pig m.l.d.	20 to 35 m.l.d.	0.50	16	Negative		"
Scarlet fever (7)	150 million skin test doses (2)*			15.2	0.71		"
" " (6)	18 million skin test doses	30,000 skin test doses	0.23	14.0			"
Streptolysin (5)	3,000 hemolytic units (2)†			16.8	2.34		"
Tetanus (8)	6.4 million mouse m.l.d.	12,000 m.l.d.	0.30				
<i>Clostridium botulinum</i>	30 million mouse m.l.d.	110,000 "	0.48	14.1		5.6	Positive

The numbers in parentheses are bibliographic references.

\* The smallest amount of toxin that will, on the average, produce an erythematous reaction, 1 cm. in diameter, in the skin of a susceptible person in 24 hours.

† The amount of hemolysin which liberates 3.75 mg. of hemoglobin from a suspension of rabbit red cells containing exactly 7.5 mg. of hemoglobin, when 1.0 ml. of hemolysin solution at pH 6.5 is added to 1 ml. of red cell suspension and incubated for 30 minutes.

fractionation at pH 6.8. In this way a more complete separation of toxin and nucleic acid-like substances was obtained. The colored impurities were best removed by precipitation at pH 4.7 to 5.0. By a repetition of these procedures at increasingly greater concentrations of toxin it has been possible to effect a remarkable degree of purification.

The criteria which showed that the toxin is a single substance are (1) constancy of activity through two successive crystallizations at 220 million mouse m.l.d. per mg. of nitrogen and (2) electrophoretic homogeneity in

the range tested from pH 3.2 through 7.0. That the toxin is a protein has been demonstrated by the properties listed in Table II.

The activity of *Clostridium botulinum* toxin places it among the group of highly active bacterial proteins (Table III). A recent preliminary report (8) places tetanus toxin in the same group. Scarlet fever toxin isolated electrophoretically is reported to be about 5 times as active as *Clostridium botulinum* toxin; however, its activity was measured by skin reactivity rather than by lethal power.

Since *Clostridium botulinum* type A toxin can now be prepared in pure form, the reaction of the toxin with formaldehyde, to produce toxoid, should be studied further. A toxoid produced in this manner would be pure enough for detailed study of its structure and composition. In addition such a toxoid should be free of bacterial protein. Preliminary work<sup>9</sup> has shown that toxoid produced from purified toxin is highly antigenic in mice.

#### SUMMARY

A protein has been isolated from the *Clostridium botulinum* type A cultures and has been found to have the biological and immunological properties of the toxin. It behaves like a globulin with an isoelectric point of pH 5.6 and a total nitrogen of 14.1 per cent. It crystallized readily in 0.10 to 0.30 saturated  $(\text{NH}_4)_2\text{SO}_4$  at 4°, forming small needle-like crystals. Twice crystallized toxin contained  $220 \times 10^6$  mouse m.l.d. per mg. of nitrogen.

The authors are greatly indebted to Dr. Norman Weissman, through whose cooperation the chemical analyses were made possible, and to Dr. Dennis W. Watson for many valuable suggestions.

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# PRELIMINARY STUDIES ON THE PRODUCTION OF $\alpha$ -KETOGLUTARIC ACID BY *PSEUDOMONAS* FLUORESCENS

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Studies in this Bureau (1) and elsewhere (2) have demonstrated that some species of *Pseudomonas* are capable of oxidizing glucose in good yield to 2-ketogluconic acid. It was of interest to us, in our search for fermentation products of industrial value, to investigate the compounds formed when *Pseudomonas* fermentations are allowed to proceed to a point where the 2-ketogluconic acid is completely metabolized. Such a fermentation in rotating drums with *Pseudomonas fluorescens* (Northern Regional Research Laboratory No. B-6) gave us a considerable amount of a crystalline calcium salt difficultly soluble in water. The elementary analysis ( $C_5H_4O_5Ca$ ) and the melting point of the 2,4-dinitrophenylhydrazone ( $226^\circ$ ) suggested that the compound was calcium  $\alpha$ -ketoglutarate. This was confirmed by conversion of the salt to an acid shown to be identical with synthetic 2-ketoglutaric acid.

It has been possible consistently to obtain yields of 16 to 17 gm. of  $\alpha$ -ketoglutaric acid from 100 gm. of glucose. Although much has been written (3) on the rôle of  $\alpha$ -ketoglutaric acid in the breakdown of carbohydrates, we believe this to be the first time that this acid has been reported as one of the main products of a carbohydrate metabolism. We hope to throw some light on the mechanism of the formation of the  $\alpha$ -ketoglutaric acid by an investigation of the other acids produced in the fermentation. We are now studying the factors which influence the yield of  $\alpha$ -ketoglutaric acid and are surveying other species of *Pseudomonas* for their ability to produce this substance.

## EXPERIMENTAL

*Fermentation Studies*—The fermentation was carried out in a rotary aluminum fermenter of the type previously used for the production of the ketogluconic acids (4). The inoculum was prepared by growing *Pseudomonas fluorescens* (Northern Regional Research Laboratory No. B-6) on a medium of the following composition: glucose 50 gm., peptone 5 gm.,

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dry yeast extract 5 gm., urea 2 gm., calcium carbonate 10 gm., lard oil 1.5 gm., and distilled water 1 liter. The culture was incubated at 30° with aeration through aloxite stones at a rate of 1 ml. of air per 1 ml. of culture solution per minute. After 48 hours, a 100 ml. portion was used to inoculate a drum containing 3 liters of culture solution of the following composition: glucose 289 gm.,  $\text{KH}_2\text{PO}_4$  1.8 gm.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.75 gm., urea 6 gm.,  $\text{CaCO}_3$  81 gm., corn steep liquor 15 ml., lard oil 5 ml., and distilled water 3 liters.

During the entire fermentation in the drum the temperature was maintained at 25°, the rotation rate at 12 R.P.M., and the pressure at 30 pounds per sq. in. The rate of air flow was held at 1200 ml. per minute until all the glucose was converted into 2-ketogluconic acid, as shown by the analytical method developed by Stubbs *et al.* (4). This phase of the fermentation required 48 hours. At this point the air flow was reduced to 600 ml. per minute, and the fermentation continued until the culture liquor showed no more reducing power. 64 hours were required for this second phase.

A portion (100 ml.) of the culture liquor was then filtered through an asbestos pad. Concentration of the filtrate *in vacuo* gave 3.27 gm. of crude calcium salts. The amount of  $\alpha$ -ketoglutaric acid in this mixture was estimated from the weight of 2,4-dinitrophenylhydrazone produced (5). The following conditions were found to give values of 96.2, 97.8, and 95.0 per cent of the theoretical when applied to pure calcium  $\alpha$ -ketoglutarate. To 50 mg. of the calcium salt were added 2.5 ml. of 2 N HCl in which 60 mg. of 2,4-dinitrophenylhydrazone had been dissolved on the steam bath. After 1 hour at room temperature, the reaction mixture was filtered through a Gooch crucible with 3 ml. of 2 N HCl and 3 ml. of water for transferring and washing the yellow precipitate. The crucible was dried at 110° for 1 hour and the amount of  $\alpha$ -ketoglutaric acid was calculated from the weight of the hydrazone.

With this method of analysis, the 3.27 gm. of calcium salt were found to correspond to 1.62 gm. of  $\alpha$ -ketoglutaric acid, which is a weight yield of 16.8 per cent of the free acid, based on the 9.64 gm. of glucose supplied. Since 1.62 gm. of  $\alpha$ -ketoglutaric acid are equivalent to 2.04 gm. of calcium  $\alpha$ -ketoglutarate, this salt represents 62.4 per cent of the total calcium salt produced in the fermentation.

A duplicate fermentation resulted in a weight yield of 16.0 per cent  $\alpha$ -ketoglutaric acid. This corresponds to a theoretical yield of 19.7 per cent if a mole of glucose can result in a mole of the acid. If, on the other hand, the  $\alpha$ -ketoglutaric acid is formed through the condensation of pyruvic and oxalacetic acids, the yield is 39.4 per cent of the theoretical.

Preliminary studies have shown that rates of air flow of 400 and 800 ml.

per minute during the second phase of the fermentation reduce the yield of  $\alpha$ -ketoglutaric acid to about one-half that found at 600 ml. per minute. It has also been possible to demonstrate by means of ether extraction that no  $\alpha$ -ketoglutaric acid is formed when the fermentation is carried only to the point of maximum production of 2-ketogluconic acid.

*Identification of  $\alpha$ -Ketoglutaric Acid*—Culture liquor obtained as described at the beginning of the preceding section was filtered after treatment with decolorizing carbon and Celite. After standing overnight, the filtrate deposited fine needles which were filtered off. Recrystallization of 2.0 gm. of these fine needles from 50 per cent alcohol gave 1.30 gm. of colorless needles which were dried *in vacuo* at 100° for analysis.

Analysis— $C_5H_7O_4Ca$  (184). Calculated. C 32.59, H 2.19, Ca 21.75  
Found. " 32.8, " 2.43, " 21.6

The calcium salt yielded a 2,4-dinitrophenylhydrazone, melting at 226–227° (decomposition).

The calcium salt (184 mg.; 1 mm) was warmed at 60° in 25 ml. of water containing 126 mg. (1 mm) of oxalic acid dihydrate. Centrifugation and lyophilization gave 137 mg. of free acid, which melted at 115–116° after crystallization from ethyl acetate-petroleum ether. This compound was shown by mixed melting point test and x-ray diffraction patterns to be identical with pure synthetic  $\alpha$ -ketoglutaric acid (m.p. 115–116°). The melting point of the 2,4-dinitrophenylhydrazone of the synthetic acid (227–228°) was not depressed by admixture with the derivative of the natural acid.

#### SUMMARY

A strain of *Pseudomonas fluorescens* has been found capable of producing 16 to 17 gm. of  $\alpha$ -ketoglutaric acid for each 100 gm. of glucose supplied in a rotary fermenter.

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# BIOLOGICAL CONVERSION OF INOSITOL INTO GLUCOSE\*

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The fact that the widely distributed natural product, *meso*-inositol, is isomeric with the common hexoses has provoked many attempts to demonstrate the glucogenic nature of inositol in animals. The results of early investigators indicate that when inositol is injected in large doses into animals a maximum of about 50 per cent is excreted unchanged (1). Attempts to relate the fate of the remainder of the inositol to the carbohydrate of the body have been numerous and inconclusive. After administration of inositol to diabetic or phlorhizinized animals no increase in sugar excretion could be shown by Külz (2), while Greenwald and Weiss found a slight increase in the G:N ratio (3). The search for newly formed glycogen in the livers of previously fasted animals given inositol produced negative results in all cases (2, 4, 5). Anderson (6) found no increase in the respiratory quotient after inositol administration. The results of Oppenheimer (7), Embden and Griesbach (8), and Griesbach and Oppenheimer (9) indicate that, unlike glucose, inositol does not bring about lactic acid formation when perfused through surviving livers. On the other hand, Starkenstein (10) showed an increase in lactic acid simultaneous with inositol disappearance in muscle and liver mince, and Mayer (11) isolated lactic acid from the urine of rabbits given inositol subcutaneously. In summarizing these various results Needham (1) finds no proof of the conversion of inositol into glucose in animals.

The recent elucidation of the configuration of *meso*-inositol, which disclosed the possibility that cleavage between a specific pair of carbon atoms would give rise to a molecule of the configuration of *d*-glucose (12), has stimulated a reinvestigation of this problem by more sensitive techniques.

Only if relatively massive amounts of a test substance are rapidly converted into glucose in the animal body will this conversion significantly influence the quantity of glucose in the urine of a diabetic animal or the quantity of glycogen in the liver of a previously fasted animal. If, however, the test substance could be stably labeled with isotope, its conversion to glucose even in small amounts might be detectable by the appearance of

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isotope in the glucose excreted or the glycogen stored. Our experience with deuterio-glucose and deuterio-glycogen is in accord with the idea that the hydrogen atoms bound to carbon in these molecules are both chemically and biologically stable (13). It was therefore determined to prepare a sample of *meso*-inositol in which the carbon-bound hydrogens were labeled with deuterium, to administer this material to suitable experimental animals, and to investigate the isotopic composition of the glucose excreted in the urine and of the glycogen deposited in the tissues of these animals.

The preparation of *meso*-inositol by the catalytic hydrogenation of hexahydroxybenzene has been reported by Wieland and Wishart (14). The unique or preponderant formation of one out of nine possible isomers was surprising, but the method seemed ideally suited to the present requirements and was consequently investigated. Noteworthy incongruities in the original description are the fact that at one point the catalyst is referred to as Pd and at another as Pt and the statement that the  $H_2$  uptake was greater than that required by theory. We have made several attempts to repeat this synthesis, investigating both Pt and Pd catalysts at several temperatures and in various solvents, but in no case could a pure product identifiable as *meso*-inositol be isolated.

We therefore sought and found conditions under which the carbon-bound hydrogen atoms of inositol would undergo exchange with deuterium of the solvent. After prolonged shaking of *meso*-inositol in  $D_2O$  solution with Pt catalyst at 130–150°, but little decomposition was found to have occurred and there was no evidence of altered configuration. Some but not all preparations of Pt catalyzed an exchange of carbon-bound hydrogen. In aqueous acetic acid no exchange occurred, and the addition of alkali, though increasing the decomposition, did not augment the exchange. Even when exchange did occur, it was notably slow. After 1 week to 1 month of shaking inositol with Pt catalyst in  $D_2O$ , deuterium had entered only between 4 and 17 per cent of the carbon-bound positions of inositol.

From such an exchange reaction a sample of inositol was obtained which, after exhaustive removal of deuterium from its hydroxyl positions, contained 1.75 atom per cent of deuterium. This product has been used in the animal experiments to be described. In each case it was administered by intraperitoneal injection in view of the described intestinal disturbance after oral administration of inositol (2, 6). Relatively large amounts were given because of the abundant urinary excretion of injected inositol (5, 10). Two of the test animals were glucosuric rats, one rendered so with phlorhizin, the other with alloxan. The third experiment involved the study of glycogen isolated from previously fasted rats.

2 gm. of deuterio-inositol were injected in divided doses over 7½ hours into a fasted phlorhizinized rat; the urine was collected for 24 hours, during

which time 2 gm. of glucose were excreted. To minimize dilution of any isotope present in this glucose, it was isolated as potassium gluconate; this material contained 0.116 atom per cent D. Whereas it was expected that conversion to potassium gluconate would effect satisfactory separation of urinary glucose from urinary inositol, the possibility was entertained that the isotope apparently present in the gluconate was actually in contaminating inositol. The gluconic acid was therefore converted into its benzimidazole derivative (15) which was predicted to contain  $0.116 \times 11/16 = 0.080$  atom per cent D. The finding of 0.072 atom per cent D in this derivative confirms our conclusion that the deuterium was indeed present in the urinary glucose excreted by the rat. As the deuterium content of the body fluids was essentially normal, the source of the deuterio-glucose must have been the deuterio-inositol that had been injected.

Fischer has shown the configurational possibility of *d*-glucose arising from *meso*-inositol by simple ring fission (12). If the deuterio-glucose in the present experiment is pictured as having arisen in this way, the potassium gluconate derived from such glucose molecules should have contained  $1.75 \times 12/6 \times 5/11 = 1.59$  atom per cent D. As the potassium gluconate isolated from the urine contained 0.116 atom per cent D,  $0.116/1.59 \times 100 = 7$  per cent of the urinary glucose may be accounted for as having arisen from inositol. This is a minimum figure, in that inositol may have been converted into glucose by more elaborate procedures involving the loss of isotope. Since 2 gm. of glucose were excreted during the period of study, at least 2 gm. must have been synthesized, or at least 140 mg. of glucose were formed from the inositol injected. Of the 2 gm. of inositol injected, therefore, a minimum of 7 per cent was utilized for the formation of glucose.

An attempt was made to demonstrate similar formation of glucose from inositol in a rat made diabetic with alloxan. Unfortunately the quantity of urinary glucose in the period following the injection of inositol was small, necessitating its isolation as the osazone and further dilution of the sample prior to its combustion for D analysis. The probable error of the analysis thus became too large to permit the attachment of any significance to the analytical figure (0.02 atom per cent D).

In a third experiment inositol was given to six previously fasted rats which were killed 3 hours thereafter. In accord with the findings of others (2, 4, 5), practically no glycogen was recovered from the livers. Abundant glycogen was found in the muscles, but it proved to contain only 0.020 atom per cent D, which is too low a value for interpretation.

We believe our evidence shows clearly that, at least in the phlorhizinized rat, inositol serves as a precursor for glucose. When compared with other glucogenic substances it is apparently not a very efficient precursor, as is

indicated by the small quantity of glucose that could be shown to arise from the inositol administered. If, after generous administration, only some 140 mg. of inositol are each day converted into glucose by the rat, it is not surprising that its glucogenic nature should have escaped detection by earlier workers. An increment in urinary glucose of this order of magnitude would, in the conventional type of experiment, be scarcely significant.

#### EXPERIMENTAL

*Attempts at Synthesis*—A number of attempts were made to synthesize *meso*-inositol from hexahydroxybenzene and related starting materials, according to the conditions of Wieland and Wishart (14) and with variation in the temperature, solvent, and catalyst. Triquinone and hexahydroxybenzene were prepared from hydroquinone by a series of reactions described by Nietzki and Benckiser (16) and Henle (17). Part of the hexahydroxybenzene was purified by repeated recrystallization from concentrated HCl containing  $\text{SnCl}_2$ , and the remainder was converted to hexahydroxybenzene hexaacetate. The hexaacetate could not be reduced at atmospheric pressure in the presence of platinum catalyst (Baker) either at room temperature or at  $100^\circ$  with acetic anhydride, acetic acid, or dioxane as solvent. Reduction of hexahydroxybenzene itself or of triquinone with platinum or palladium catalyst in water at various temperatures between  $25^\circ$  and  $100^\circ$  resulted in hydrogen uptake, often more than required by theory for inositol formation, but no pure product identifiable as *meso*-inositol could be isolated.

*Preparation of Deuterio-Inositol by Exchange with Heavy Water*—A number of attempts were made to introduce deuterium into carbon-bound positions in inositol by exchange reactions. Carefully purified *meso*-inositol was sealed into bulbs in the presence of platinum catalyst in neutral, acid, or alkaline  $\text{D}_2\text{O}$ , and the bulbs were shaken 1 to 4 weeks at  $130$ – $150^\circ$ . *meso*-Inositol isolated from the solution was analyzed for deuterium. In three out of a total of twelve trials, carbon-bound deuterium was introduced. In all trials in which the platinum oxide catalyst was reduced by bubbling  $\text{H}_2$  through the mixture of inositol, catalyst, and solvent, no exchange occurred. Better results were obtained when the catalyst was previously completely reduced by shaking in water under an atmosphere of  $\text{H}_2$  and then transferred to the exchange bulb containing the inositol. This has been taken to mean that complete reduction of the catalyst is essential. In such trials no exchange was found to have taken place with 50 per cent acetic acid as solvent, whereas it did occur with distilled water or  $0.1 \times \text{NaOH}$ . In the best two trial runs in which low concentrations of  $\text{D}_2\text{O}$  were employed, 17.0 per cent of the C-bound hydrogen atoms was exchanged in

water and 17.4 per cent was exchanged in dilute alkali in 1 week. When water was used as solvent, *meso*-inositol was recovered in good yields and only slight decomposition was noted, whereas in dilute alkali considerable decomposition took place and less *meso*-inositol could be recovered. One attempt was made to introduce deuterium by irradiating a solution of inositol in  $D_2O$  with x-rays. When 1 gm. of inositol in 20 cc. of  $D_2O$  was irradiated with about 4000 Roentgen units per minute for 1 hour, no stable D was introduced.<sup>1</sup>

The sample of deuterio-inositol used for the biological experiments was prepared by exchange in 99 atom per cent  $D_2O$ . 8 gm. of inositol, sealed in a bulb with 25 gm. of  $D_2O$  and 1.6 gm. of Pt catalyst, were shaken at 130–150° for 30 days. The solution became light brown in color. The recovered solvent was found to contain 89.5 atom per cent  $D_2O$ . Water was repeatedly added to the residue and evaporated away to remove all excess D from the hydroxyl positions of the inositol. Inositol was isolated by precipitation from water with ethanol and recrystallized from aqueous ethanol. 6.41 gm. of a compound were recovered with properties identical with those of the pure *meso*-inositol originally used.

Calculated, C 40.0, H 6.67; found, C 39.95, H 6.44

Starting material, m.p. 224–226°; isolated material, m.p. 225–226°

Mixed m.p. 224–226°

The inositol contained 1.75 atom per cent of D, all of which must have been bound directly to C atoms. The concentration of D in such positions was therefore  $1.75 \times 12/6 = 3.50$  atom per cent D, and consequently,  $3.50/89.5 \times 100 = 3.9$  per cent of all the carbon-bound hydrogen had undergone exchange.

*Inositol Administration to Alloxan-Diabetic Rat*—An adult white rat was made diabetic by the injection of 15 mg. of alloxan monohydrate per 100 gm. of body weight. It was found to excrete 6 to 9 gm. of glucose per day when on a diet of Rockland rat pellets. The rat was fasted for a 9 hour preliminary period, after which 2 gm. of deuterio-inositol in 16 cc. of sterile water were injected intraperitoneally in four equal portions at 2 hour intervals. Drinking water was given *ad libitum* but no food was allowed; the urine was collected for 24 hours after the first injection. Since only 78 mg. of glucose were present in this urine, isolation as glucosazone was resorted to, a procedure that necessarily diluted any deuterium that may have been initially present in the glucose, and the 68 mg. of recrystallized osazone obtained had to be diluted further for analysis. The analytical value of 0.02 atom per cent D in this sample was too low for interpretation.

<sup>1</sup> We wish to thank Dr. T. C. Evans of the Department of Radiology for the administration of the x-rays.



Therefore, in the hope of obtaining a larger urinary excretion of glucose, a phlorhizinized rat was next investigated.

*Inositol Administration to Phlorhizinized Rat*—An adult white rat, fasted for a preliminary 9 hour period, was made glucosuric by the hypodermic injection of a sesame oil emulsion of 0.01 gm. of phlorhizin per 100 gm. of body weight. 2 gm. of deuterio-inositol in 16 cc. of water were injected intraperitoneally in four doses at intervals of  $2\frac{1}{2}$  hours. The urine collected for 24 hours after the first inositol injection was found to contain 2.0 gm. of glucose. A sample of water distilled from the urine contained 0.004 atom per cent D. The urinary sugar was oxidized to gluconic acid and 0.916 gm. of potassium gluconate was isolated. Part of the K gluconate was reprecipitated from water with ethanol and was found to contain 0.116 atom per cent D. The remainder of the K gluconate was converted to its benzimidazole derivative (15). The gluco-benzimidazole, after recrystallization from water, contained 0.072 atom per cent D, as compared with the value of 0.080 predicted on the basis of the D analysis of the K gluconate.

*Inositol Administration to Normal Fasted Rats*—After six adult rats had been fasted for 17 hours, each was injected intraperitoneally with 370 mg. of deuterio-inositol in 3 cc. of water. The rats were killed 3 hours later by a blow on the head and glycogen isolated from the total combined livers and from the carcasses (18). Some unabsorbed fluid was noted in the peritoneal cavity. A sample of body water contained 0.014 atom per cent D. There was only a trace of glycogen in the livers. From the eviscerated carcasses 1.13 gm. of glycogen containing 0.020 atom per cent D were obtained.

#### SUMMARY

*meso*-Inositol containing an excess of deuterium in its carbon-bound positions has been prepared by platinum-catalyzed exchange with  $D_2O$  at elevated temperature.

The administration of deuterio-inositol to a phlorhizinized rat resulted in the appearance of significant concentrations of D in the urinary glucose.

It is concluded that conversion of *meso*-inositol into glucose takes place in the body of the rat.

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# BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

## II. THE PEPSIN DIGESTION AND RECOVERY OF HUMAN $\gamma$ -GLOBULIN\*

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The main plasma proteins may be roughly classified in terms of their solubilities, and of their electrophoretic and sedimentation behavior, as albumin, the various globulins, and fibrinogen. Making use of the knowledge gained from the isoelectric and neutral salt precipitation methods for which their Laboratory is well known, Cohn *et al.* (1) have developed further the low temperature alcohol precipitation methods of Mellanby (2), of Hardy and Gardiner (3), and of subsequent investigators to separate the plasma proteins into these components. The process developed by Cohn and his coworkers according to which human plasma has been treated during the war years separates the plasma proteins into four initial fractions (4). They are as follows: Fraction I, fibrinogen; Fraction II + III,  $\beta$ - and  $\gamma$ -globulins; Fraction IV,  $\alpha$ -globulins; and Fraction V, albumin.

Of these, the fraction called Fraction II + III contains the immune globulins, prothrombin, and the isohemagglutinins. This fraction has been subdivided in the interest of further separation and concentration of components possessing specific physiological activities. Methods have been developed by Cohn and his associates to provide for the concentration of prothrombin in what they have designated as Fraction III-2, the isohemagglutinins in a Fraction III-1, and the immune globulins in Fraction II. By their Method 3, roughly one-half of the  $\gamma$ -globulin is removed in highly purified form as Fraction II. On the other hand, Fractions III-1 and III-2 are far from homogeneous when studied by electrophoresis. Enders (5) reports that the greater part of the antibodies not accounted for in Fraction II is contained in Fraction III-1. The available evidence indicates that many of the antibodies are  $\gamma$ -globulins; this finding is not unexpected when it is realized that Fraction III-1, when prepared by Method 3, contains about 27 per cent of  $\gamma$ -globulin as revealed by electrophoresis.

\* This work has been carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin. A portion of the subject matter of this report has been used in Invention Disclosure 2614 of the Office of Scientific Research and Development (1945).

Since the recovery of as much  $\gamma$ -globulin as possible is desirable, one may seek either (1) to extract additional  $\gamma$ -globulin from the Fraction III-1 residues, or (2) to improve the theory and practice of the separation of Fraction II + III so that the yields of  $\gamma$ -globulin in Fraction III-1 will decrease and correspondingly increase in Fraction II.

Of these alternatives the second one is more important by far, because one can always succeed with the first when the principles involved in the separation of  $\beta$ - from  $\gamma$ -globulins are understood. Substantial progress with each problem has been made in this Laboratory.

Actually, two distinct methods for the extraction of  $\gamma$ -globulin from pastes containing  $\beta$ -globulin as well have been developed. Although the material available for this developmental work was often the Fraction III-1 as obtained by Method 3C (4), it is emphasized that these methods are by no means restricted to treatment of this particular fraction for the recovery of  $\gamma$ -globulin. Thus, these pastes may be the initial Fraction II + III or other subfractions corresponding to the Fraction III-1 of Method 3C from which some of the original  $\gamma$ -globulin has been removed. One of the methods, to be described in this report, makes use of a pepsin digestion step in a system of ionic strength 0.025 to 0.030. The second procedure is to be considered in another place.

### *Peptic Digestion of Human $\gamma$ -Globulin*

A study of the conditions for the peptic hydrolysis of human  $\gamma$ -globulin to yield immunologically active fragments has been carried out by Bridgman (6). This work has been preceded by similar studies with diphtheria antitoxin (7), with horse antipneumococcus antibody (8), and with bovine serum globulin (9). It has been customary in this Laboratory to analyze such globulin digests in terms of the relative amounts of globulin of unchanged sedimentation constant and of apparent halves and quarters of the normal globulin molecule, together with dialyzable material. Bridgman (6) has discussed the manner of cleavage by pepsin of the human  $\gamma$ -globulin molecules, and Pappenheimer and Petermann (7-9) also have considered animal globulin systems from this point of view. To gain information on this point the globulin digest systems were studied in the Svedberg oil turbine, high velocity ultracentrifuge and their molecular mass spectra were determined by analysis of line displacement-distance diagrams. Sedimentation constants were calculated in the usual way. Normal human  $\gamma$ -globulin has a sedimentation constant,  $s_{20}$ , of about 7 Svedberg units. The so called half molecules have  $s_{20} \cong 6$  Svedberg units and the corresponding quarter molecules  $s_{20} \cong 4$  Svedberg units. For convenience the components of a digest are referred to as  $s^7$ , globulin of normal size;  $s^6$ , globulin of apparent half size; and  $s^4$ , globulin of apparent

quarter size. Protein fragments small enough to pass through Visking tubing are too small for satisfactory ultracentrifuge analysis, and, accordingly, no attempt has been made to study their size distributions. In adapting the method to large scale operations, it has been necessary to devise chemical measures of the extent of digestion, to consider the removal of pepsin from the digest systems, and to make immunological assays of the product.

When the serum globulins are cleaved by pepsin at hydrogen ion concentrations well removed from the pepsin optimum, at any one pH the system reaches an apparent equilibrium, the relative amounts of the normal, half, and quarter molecules and of smaller fragments being characteristic of the particular globulin used, and of the particular pH. Thus, at pH 4.4 and 0°, increasing amounts of dialyzable fragments are formed from human  $\gamma$ -globulin (6), but the remaining protein does not contain any appreciable proportion of half molecules. At pH 3.5 and 0° from 60 to 70 per cent of the non-dialyzable protein is left as half molecules. For other globulins, such as horse diphtheria antitoxin and beef serum globulins, the maximum yield of half molecules is obtained at pH 4.0 (7). Horse antipneumococcus globulin can be split into halves at pH 4.5 (8). Temperature and pH are interdependent in some respects.

Thus the proportion of half molecules present in a new serum globulin digest cannot be ascertained from the amount of small fragments formed. However, once the ratio of half molecules to small fragments has been determined for any species of globulin digested at a given pH and temperature, it should be possible to set up a relationship between pH and extent of formation of half molecules.

Determination of the per cent decrease in the nitrogen in solution after dialysis is satisfactory in the analysis of completed digests. For the control of a digestion in progress, some more rapid method is needed. It has been shown by Haugaard and Roberts (10) that the proportion of a protein digest soluble in 10 per cent trichloroacetic acid corresponds to the amount which will pass through Visking casing. The increase in material soluble in 10 per cent trichloroacetic was therefore set up as a criterion of the extent of digestion. Again, some procedure quicker than a nitrogen analysis was sought. Since the colorimetric determination of "tyrosine" (substances which reduce the Folin-Ciocalteu reagent) may be used as a method of pepsin assay (11), this method was adapted to the measurement of the tyrosine soluble in 10 per cent trichloroacetic acid. The procedure does not involve any further digestion, and can be carried through in a very short time.

The digestion procedure for human  $\gamma$ -globulin employed was substantially that developed by Bridgman (6), with modifications described below. All operations were conducted at 0-2° unless otherwise specified.

The Fraction II globulins used in this work were contained in 20 per cent solution. For the digestion, an equal volume of water was added to the protein. Then 0.05 *N* HCl was added from a capillary-tipped siphon, with mechanical stirring, until the pH of the solution was about 3.8. With the larger digests, stirring was continued for at least an hour to permit the removal of any carbon dioxide formed by acidification of bicarbonate. Pepsin was then added, 0.10 unit of crystalline pepsin per gm. of protein, previously diluted to about 0.3 unit per ml. As soon as the pepsin had been thoroughly mixed with the globulin, 1 ml. of the mixture was taken for determination of non-protein nitrogen and tyrosine. The addition of HCl was then continued until pH 3.7 to 3.6 was attained. The measurement of pH is carried out at 25°.

At least once a day the pH of the digest was measured, and, whenever necessary, more acid was added to restore the pH to 3.6. The course of the digestion was followed by daily non-protein tyrosine determinations and had usually progressed far enough in about 65 hours. At this point, the concentration of  $\gamma$ -globulin was 4.5 to 5.0 per cent. A final sample was taken and the amount of non-protein nitrogen and tyrosine per ml. was determined. The digest was then neutralized with 0.05 *N* NaOH to pH 5.0. Some turbidity, presumably due to the small amount of  $\beta$ -globulin present, appeared. Enough Super-Cel to give a 0.5 per cent suspension was added, and the mixture was stirred for 2 hours. It has been found that Super-Cel, used under these conditions, is one of several adsorbents which are effective in removing all but very small traces of the enzyme.

Sufficient 50 per cent (by volume) ethanol was then added to bring the alcohol concentration to 8 per cent. The alcohol was cooled to just above its freezing point in a dry ice-alcohol bath, and was added slowly with vigorous stirring. The temperature of the digest was kept at just above its freezing point. The precipitate was centrifuged off at -2°, and the digest mixture brought to pH 6.0 by the addition of *M* NaHCO<sub>3</sub>. 95 per cent ethanol, cooled to -18°, was then added slowly with stirring, until the alcohol concentration reached 40 per cent. The solution was kept at -5° to -7° throughout the addition. The globulin precipitate was removed by centrifugation at -5°, suspended in ice water, shell-frozen, and dried *in vacuo*.

The results of some typical pepsin digestion experiments with Fraction II preparations are shown in Table I. In Experiments 35-1 and 29-2 an insufficient amount of pepsin preparation was used and little digestion was obtained. For molecular mass spectrum analysis, the final powder was dissolved in 0.15 *M* NaCl in about 1.8 per cent solution, and studied in the ultracentrifuge. Line displacement-distance in cell diagrams obtained

after about 120 minutes at 60,000 R.P.M. was resolved by the usual procedure. Some typical diagrams are shown in Fig. 1. The sedimentation diagram for Experiment 35-1 (see Fig. 1, A) is that of unchanged globulin. It is included as a control experiment, to show that the acid alone causes no permanent change in the sedimentation characteristics of the globulin. An early stage in the enzymatic cleavage (Experiment 29-2) is shown in Fig. 1, B. These experiments demonstrated the need for precise control of the process.

TABLE I  
*Typical Pepsin Digestion Experiments with Human  $\gamma$ -Globulin Preparations*

Experiment No.	Globulin	Pepsin	Time	Non-protein N	Tyrosine	Sedimentation, per cent protein		
						$s^2$	$s^4$	$s^2$
	gm.	unit per gm.	hrs.	per cent	mg. $\times 10^4$ per ml.			
35-1	0.5	0.025	18			100.		
29-2	66	0.20	22			61	24	15
29-3	1	0.10	24	9	12.7			
			47	10	15.6	24	52	24
			72	15	21.6	19	59	22
29-4	64	0.10	22	10	13.7			
			47	14	19.2			
			66	13	19.4	14	35	51
291K-1	1	0.10	65		28.1	14	70	16
291K-2	64	0.10	0	0	0			
			43	10	13.2			
			65	13	17.4	25	51	24
291K-3	60	0.10	0	0.8	2.2			
			66	13	22.3			
			90	15	25.1	17	66	17
291K-4	192	0.10	0	0.8	2.0			
			63	14	24.9	17	63	20

The sort of diagram obtained in a successful digestion experiment is shown in Fig. 1, C. About equal amounts of components larger and smaller than halves are present. If the digestion is carried farther, in the hope of splitting all of the globulin, much material is lost by cleavage into non-protein fragments.

*Large Scale Recovery of Human  $\gamma$ -Globulin from Pastes Containing  $\beta$ - and  $\gamma$ -Globulins; Enzyme Digestion Method*

In the earlier commercial methods for the recovery of  $\gamma$ -globulin from Fraction II + III, roughly one-half of the  $\gamma$ -globulin was precipitated in the Fraction III-1 residue, together with a corresponding quantity of



the antibody activity. In order to separate this  $\gamma$ -globulin in a form which is sufficiently free of the accompanying  $\beta$ -globulin, the Fraction III-1 paste must be treated under new conditions such that there is an appreciable difference in solubility between the  $\gamma$ - and the several  $\beta$ -globulins.

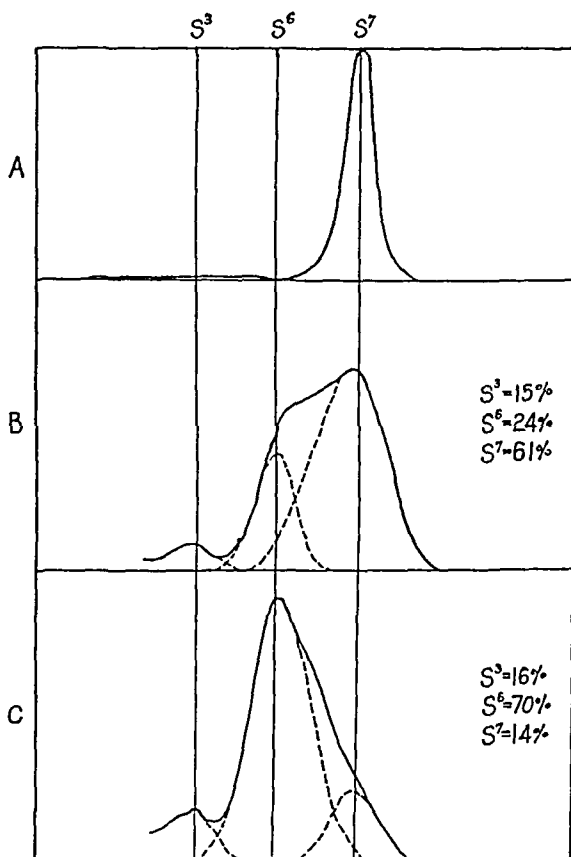


FIG. 1. The digestion of Fraction II globulins by pepsin at pH 3.7. A, Preparation IIGS-35, 0.025 unit of pepsin per gm. of globulin; B, Preparation IIGS-29, 0.020 unit; C, Preparation IIGS-291K, 0.100 unit.

It has been demonstrated that human  $\gamma$ -globulin can be treated with pepsin to give a system in which molecules of one-half of the normal size predominate, without loss of important antibody activity as judged by the usual immunological assays. From the decrease in molecular size, it might be expected that, if the  $\gamma$ -globulin in the Fraction III-1 paste is preferentially digested, its solubility might be increased as compared to that of the accompanying  $\beta$ -globulins. Preliminary solubility determina-

tions with  $\gamma$ -globulin systems, undigested and digested, showed a distinct difference in favor of the latter at  $-5^{\circ}$ , the temperature at which the separation of  $\beta$ - and  $\gamma$ -globulin is carried out. In the Fraction III-1 mixture, the  $\beta$ -globulins may also be somewhat digested, but apparently their solubility is not appreciably increased. It therefore appeared feasible, by the inclusion of a pepsin digestion step, to develop a process for the large scale recovery of  $\gamma$ -globulin from these Fraction III-1 residues.

The process consists of a partial digestion of Fraction III-1 by pepsin at pH 3.5, followed by fractional precipitation, whereby the  $\beta$ -globulin is separated from the  $\gamma$ -globulins. Two things are to be especially noted. (1) The pepsin requirement per kilo of protein is very much higher (about 5 times) as compared to that necessary to digest nearly pure  $\gamma$ -globulin preparations, probably because the Fraction III-1 residue contains an enhanced concentration of enzyme inhibitor. (2) The favorable result obtained depends in part upon the fact that the ionic strength of the system at the time of separation of  $\gamma$ - from  $\beta$ -globulin has been reduced from the customary 0.040 to 0.029. This observation has been of far reaching consequence, because we have demonstrated that, when the ionic strength is as high as 0.040 in this system,  $\gamma$ -globulin is salted-out in copious amounts into the  $\beta$ -globulin residue. This phenomenon will be considered further in a subsequent report.

Fraction III-1 is a paste of precipitated globulin together with solvent which contains about 30 per cent protein, of which about 27 per cent is  $\gamma$ -globulin. Its alcohol content has been assumed to be 11 per cent. The pepsin used is the commercial (Parke, Davis) 1:10,000 preparation which has been fractionated once by magnesium sulfate according to the procedure used by Philpot (12). This preparation of pepsin contains only 0.10 hemoglobin unit per mg. of nitrogen, but the single treatment removes a large part of the foreign antigenic protein and further purification is considered to be too costly for large scale operation.

The description of the following procedure is based upon an operation with 1 kilo of a typical Fraction III-1 paste. Only slight modifications would be required to adapt it for use with Fraction II + III. It is to be remarked that the conditions for the actual separation of digested  $\gamma$ -globulin from the  $\beta$ -globulins are essentially those employed at the Harvard pilot plant to prepare normal serum  $\gamma$ -globulin, but with the important exception that a somewhat lower ionic strength is employed. Moreover, the salt present was largely sodium chloride rather than sodium acetate. 1 kilo of material is suspended in 1.5 liters of 0.15 M NaCl at  $0^{\circ}$  with mechanical stirring and 3 liters of 0.05 M HCl, also at  $0^{\circ}$ , are then slowly added with vigorous stirring. A solution that contains approximately 112 units of pepsin is diluted to 250 ml. with cold water and added slowly.

TABLE II  
Recovery of  $\gamma$ -Globulin from Fraction III-1 Residues; Pepsin Digestion Method

Experiment No.	Fraction III-1	Pepsin	Non-protein N	Fractionation				Pptn.				Sedimentation				Electrophoresis				Yield
				pH	$\frac{I}{2}$	Alcohol	Protein	pH	$\frac{I}{2}$	Alcohol	Protein	s <sup>1</sup>	s <sup>2</sup>	s <sup>3</sup>	per cent of total	$\gamma$ -Globulin	$\beta$ -Globulin	$\beta$ -Globulin	Albumin	
	kg.	unit per gm.	per cent			per cent	per cent			per cent	per cent	per cent of total	per cent of total	per cent of total	per cent of total	per cent of total	per cent of total	per cent of total	per cent of total	
D-1	0.4	0.625		5.0	0.05	16	2.5	6.5	0.036	48	1.0	5	57	4	34					27
D-2	0.4	0.6	24	5.1	0.037	18	2.5	6.5	0.031	42	0.9	1	46	6	47					16
D-3	0.4	0.6	15	5.0	0.04	14	2.5	6.0	0.03	38	1.0	22	40	13	26					28
D-4	0.4	0.6	21	4.9	0.047	12	2.5	6.0	0.03	30	1.0	8	50	9	33					58
D-5	0.4	0.6	22	5.0	0.034	15	2.5	6.0	0.03	30	1.0	7	53	4	31	77	19	4		
D-6	0.4	0.44†	30	5.1	0.031	15	2.5	6.1	0.027	30	1.0	2	60	8	30	74	19	4	4	31
D-7	0.4	0.22‡	22	4.9	0.034	15	2.5	6.0	0.029	30	1.0	9	63	0	28	85	11	4		29
D-8	0.4	0.22‡	26	5.1	0.035	13	2.5	5.3	0.029	27	1.0	5	53	4	38	79	17	2	2	33
DS1-1	4	0.60		5.2	0.031	15	2.5	6.1	0.027	30	1.0	13	68	0	19	90	7	3	0	165
DS1-2	4	0.60		5.2	0.034	15	2.5	6.0	0.029	30	1.0	19	50	0	31	90	8	2	0	250
DS1-3	4	0.60		5.1	0.031	15	1.2	6.0	0.026	30	0.5	34	47	4	15	89	8	3	0	300
DS2-4	4	0.48		5.2	0.027§	15	1.2	6.0	0.028	30	0.5	28	54	2	16	71	21	6	2	340
DS2-5	4	0.45		5.2	0.031	15	1.2	6.0	0.026	30	0.5	22	54	0	24	85	11	4	0	269
DS3-6	8	0.45		5.1	0.031	15	1.2	6.0	0.026	30	0.5	26	54	0	20	88	10	2	0	311
DS2-7	3	0.40		5.1		15	1.2													
DS2-8-A	2	0.506		5.1	0.028	15	1.2	6.1	0.030	15	0.5	24	61	3	12	78	17	5	0	44
DS2-8-B								6.1	0.025	20		7	63	4	26	91	6	3	0	116
DS2-9-A	2	0.48		5.1	0.028	14	1.2	6.1	0.030	14	0.5	11	63	2	24	57	35	8		50
DS2-9-B								6.1	0.028	17						72	23	5		83
DS2-9-C								6.1	0.026	20		4	60	2	34	79	13	8		49
DS2-9-D								6.1	0.023	30										59

\* The experiments in the first section were carried out in this Laboratory; the remainder, at Harvard Medical School, Boston.

† Yield expressed in gm. of protein recovered.

‡ Crude pepsin used.

§ Diluted with 0.036 M sodium acetate, pH 5.2, instead of 0.04 M NaCl.

Sufficient 0.05 M HCl (about 500 ml.) to bring the pH of the system down to 3.5 is then added. (All pH measurements are made at 25°.) The digesting system is allowed to stand at 0–1°; its pH tends to rise slowly. Every few hours enough dilute HCl is added to return the pH to  $3.5 \pm 0.1$ . At this pH the digestion has usually proceeded far enough in 15 to 25 hours, when a non-protein tyrosine determination is made. A value in the neighborhood of  $30 \times 10^{-4}$  milliequivalent of tyrosine per ml. of digest, or per 8 to 9 mg. of original protein, indicates a sufficient degree of digestion. The pH of the digest is now adjusted to 5.10 to 5.20 by the addition of 0.05 M NaOH, about 3.5 liters being needed. About 50 gm. of Super-Cel suspended in 0.25 liter of water are added to the system and the suspension is stirred for 2 hours, when 10 liters of 0.04 M NaCl are added. The alcohol concentration is then increased to 15 per cent by the addition of about 7.5 liters of 53.3 per cent alcohol (cooled to about  $-5^\circ$ ). During this addition, the temperature of the system is kept just above its freezing point. After being stirred for 2 hours, the precipitate containing  $\beta$ -globulin and pepsin is centrifuged off at  $-5^\circ$ . The effluent is filtered through Super-Cel mats on ordinary filter paper in Büchner funnels. The filtrate is adjusted to pH 6.0 to 6.1 by the addition of approximately 37 ml. of M  $\text{NaHCO}_3$ . Its alcohol content is then increased to 30 per cent by the addition of precooled 95 per cent ethanol, at  $-5^\circ$  (5.77 liters of ethanol for 25 liters of effluent). The precipitate is centrifuged off at  $-5^\circ$ , suspended in an equal volume of water, shell-frozen, and dried *in vacuo*.

A summary of the experiments is given in Table II. Experiments D-1 to D-8 were carried out in this Laboratory. The pH measurements were made on cold samples. After correction to room temperature the variation in pH was between 3.55 and 3.70. Bucket centrifuges were used and the filtration step was omitted. From Experiments D-2 and D-3, it may be seen that precipitation of the  $\beta$ -globulin with 18 per cent alcohol gives a low yield of purer  $\gamma$ -globulin, while 12 per cent alcohol gives a good yield but fails to remove enough  $\beta$ -globulin. When 15 per cent alcohol is used, a desirable compromise between maximum yield and optimum purity is achieved. In Experiments D-6, D-7, and D-8, crude pepsin powder was used. The product appeared to be satisfactory, although it is possible the small amounts of fast moving material found on electrophoretic analysis may have been derived from the pepsin.

The experiments listed in the lower section of Table II were performed in the pilot plant at the Harvard Medical School, Boston, Massachusetts.

The more adequate temperature ( $0^\circ$ ) and pH ( $3.50 \pm 0.07$ ) control, the filtration procedure, and the tighter packing of precipitate achieved with the Sharples centrifuge are reflected in an improved product. The

doubling of the volume before fractionation by the addition of 0.04 M NaCl was first tried in Experiments DS1-1, DS1-2, and DS1-3. This step resulted in an appreciable increase in yield.

Experiment DS2-4 differs from the standard procedure in that the volume was doubled before fractionation by the addition of 0.036 M acetate buffer, pH 5.2. The product contained an unusually high proportion of  $\beta$ -globulins; so this modification was not repeated.

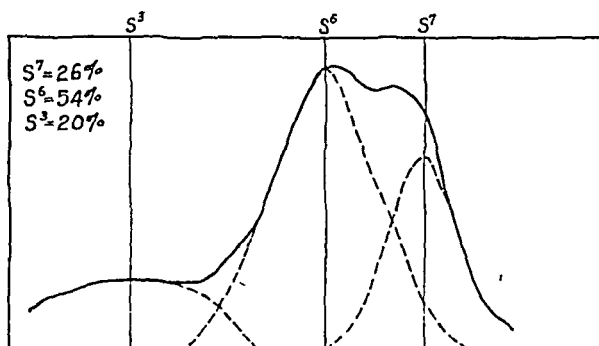


FIG. 2. Sedimentation analysis of Preparation DS3-6

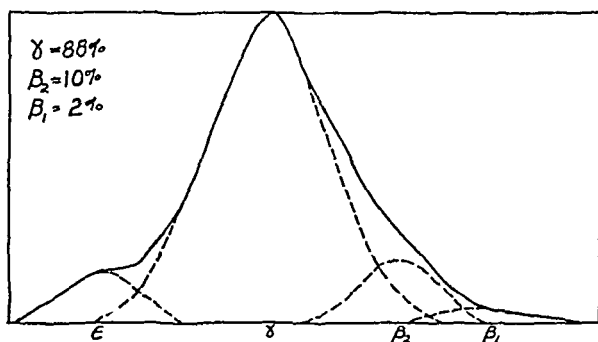


FIG. 3. Electrophoretic analysis of Preparation DS3-6

In Experiment DS2-7 the pH was buffered during the digestion by the addition of citric acid. Only 34 gm. of  $\gamma$ -globulin product were obtained from 3 kilos of paste.

The series of products obtained has been analyzed by physical, chemical, and immunological methods. Ultracentrifugal and electrophoretic analyses were made in the usual way, and the diagrams are presented as Figs. 2 and 3. Electrophoretic analyses were carried out in barbiturate-citrate buffer ( $\Gamma/2 = 0.088$ ) at pH 8.6. The results obtained with Preparation

DS3-6 may be considered as typical. The product consists of about 55 per cent half molecules with lesser amounts of normal globulin and small fragments. It is 88 per cent  $\gamma$ -globulin, with 10 per cent of  $\beta_2$ , 2 per cent of  $\beta_1$ , and no  $\alpha$ -globulin or albumin. These powders dissolve in saline to give water-clear solutions.

### Application

Although it has been shown that these experiments provide a means for the extraction of  $\gamma$ -globulin from  $\beta$ - and  $\gamma$ -globulin pastes, their greatest usefulness will come in the treatment by pepsin of human  $\gamma$ -globulin antibody preparations in order to alter the absorption rate and distribution of the antibodies in the body and to prepare the material for subfractionation to concentrate further certain of the antibodies.

TABLE III

*Immunological Assay\* of Products of Pepsin Digestion (Enders and Sullivan)*

Preparation No.	Typhoid "O"	Typhoid "H"	Diphtheria antitoxin	Influenza A		
				Complement fixation	Hirst	Mouse pro- tection
D-2	0.60	0.2	0.5		1	1.0
D-6	0.25	0.7	0.6		1	1.1
D-7	0.25	0.3	0.6		1	3.0
DS1-1, 2, 3†	1.0	1.4	1.0	1.0	2	2.7

\* Values referred to a standard fraction of  $\gamma$ -globulin (Fraction A66), as used by Enders (5).

† Results of pooled samples from three experiments (Nos. DS1-1, DS1-2, DS1-3) performed in the Harvard pilot plant.

Thus it is at once a matter of interest and importance to the immunologist to be assured that antibody activity has not been destroyed, and to consider the success of the removal of any foreign antigenic protein which may have been introduced either as or with pepsin. These two problems are dealt with successively in the paragraphs which follow.

We have been fortunate in having the aid of Dr. J. F. Enders, Miss J. C. Sullivan, and their associates, who have carried out all of the antibody assays we present in this report. Representative values for some products of both small and large scale  $\gamma$ -globulin digestion recovery experiments are given in Table III.

These preparations, all of which contain 85 to 90 per cent  $\gamma$ -globulin, have an excellent titer of most antibodies. In general the titers agree with those reported by Enders (5), although the antiinfluenza A mouse protection values are sometimes distinctly enhanced. In addition, it

was found by Dr. C. A. Janeway that data consistent with those obtained by using standard Fraction II  $\gamma$ -globulin were secured when solutions of Preparation DS1-1,2,3 were tested clinically for measles prophylaxis.

The process of digestion may involve the addition of foreign protein with the enzyme even when crystalline pepsin is used. It therefore becomes important to determine whether or not sufficient antigenic material remains in the final product to be a source of danger in a clinical use. The available results are shown in Table IV and it is apparent that it has not been possible so far to free the digested human globulin entirely from contaminating foreign protein. However, commercial digested antitoxins are no better in this respect. Also intradermal tests made with dilute digested globulins failed to reveal any significant responses, and

TABLE IV  
*Pepsin Removal Studies*

Preparation No.	Conditions	Shock-producing dose	Severity of symptoms*
		mg.	
DS1-1, 2, 3	Normal fractionation	10	3.3
DS2-4	" "	10	3.7
DS2-4a	Reprecipitated at pH 7.03	50	4.0
DS2-4b	2 times pptd. at pH 7.0	30	2.0
DS2-4b	2 " " " " 7.0	45	4.0
DS2-4b	2 " " " " 7.0	90	5.0
DIIGL-291K†	Pptd. at pH 7.0	10	0
DIIGL-291K†	" " " 7.0	100	0.6

\* Based on scale from 5 (death within 5 minutes after injection) to 0 (no symptoms).

† Twice crystallized pepsin used for digestion. Some of these assays were performed by Dr. C. A. Janeway.

skin tests performed under the direction of Dr. Janeway in Boston with a 0.05 per cent pepsin solution upon a group of fourteen children who had received an intramuscular dose of 0.1 to 0.2 ml. of Preparation DS1-1,2,3 were negative 29 days after injection. The intramuscular injections were made without the appearance of serum disease-like symptoms.

Two types of experiment were carried out to test procedures designed to remove the traces of foreign protein left in the  $\gamma$ -globulins prepared by using the pepsin digestion method. One approach consisted of a reprecipitation of the digested  $\gamma$ -globulins at pH 7.0. The second procedure was to make use of twice recrystallized pepsin in the treatment of the Fraction III-1 starting material. Products were tested by intravenous administration to guinea pigs that had been previously sensitized to our

crude pepsin preparations. The results of these experiments, shown in Table IV, suggest that digestion with crystalline pepsin gives a product free from all but a trace of foreign protein. From 60 to 70 per cent of the small amount of foreign protein remaining in the products prepared by digestion with crude pepsin may be removed by an additional precipitation at pH 7.0 with 25 per cent ethanol. Further precipitations are not correspondingly effective.

TABLE V  
*Concentration of Antibodies by Fractionation of Peptic Digests*

Preparation No.	Conditions of pptn.		Per cent of total pro- tein recovered	Immunological assays*					Sedimentation analysis		
	pH	Ethanol		Typhoid "O"	Typhoid "H"	Diphtheria anti- toxin	Influenza A		s <sup>2</sup>	s <sup>4</sup>	s <sup>2-4</sup>
							Human	Mouse pro- tection			
		per cent							per cent of total	per cent of total	per cent of total
DS2-8a	6.12	15	29	0.47	0.64	0.9	2.6	3.0	24	61	15
DS2-8b	6.12	30	71	0.42	0.88	0.82	1.2	2.5	7	63	30
IIGS-29R	Parent fraction			1.2	0.6	0.46		1.0			
DIIGS-29Ra	6.15	15	28	0.59	0.64	0.53	1.8	1.2	12	51	37
DIIGS-29Rb	6.15	17	15	0.65	0.71	0.53	1.0	1.2	10	68	22
DIIGS-29Rc	6.15	20	31	1.2	0.90	0.79	0.9	0.7	4	61	35
DIIGS-29Rd	6.15	30	26	1.9	0.95	0.61	0.9	0.8	3	73	18
DS2-4	Parent			0.83	0.55	1.0	2.2	2.0	28	54	18
DS2-4a	5.4	18	60	0.88	0.29	0.67	2.4	2.6	54	30	16
DS2-4b	6.0	18	19	0.75	0.50	1.23	2.0	2.6	67	20	13
DS2-4c	6.7	18	10	1.36	0.68	1.23	1.8	2.6	40	40	20
DS2-4d	6.7	30	11	1.35	0.68	1.23	1.8	2.0	15	54	31

\* Values referred to a standard fraction of  $\gamma$ -globulin (Fraction A66), as used by Enders (5). These data were obtained by Enders. All of the influenza A complement-fixing antibody was present in Preparation DS2-8a.

Studies on the rate and degree of absorption of antibodies, digested and undigested, after injection by various routes are in progress. It is expected that, in systems made up of predominantly smaller molecules, combination with antigen in diseased tissue and in the blood stream will be accelerated.

Since digested  $\gamma$ -globulin systems are paucidisperse, the several components may have different resistances to heat treatment and different solubilities in aqueous ethanol. The term paucidisperse has been used by Svedberg and Pedersen (13) to describe a system in which several distinct components are present. The system is not monodisperse; on the other hand the description of it as polydisperse would convey another meaning.



Also, the theories of Pauling (14) suggest that antibodies are  $\gamma$ -globulin molecules which have surface orientation of groups peculiar to their biological activity. Thus, different antibodies would have different surface properties and configurations and it is possible they may show different responses to peptic digestion. As a result of what may be termed differential digestion and subsequent chemical fractionation, additional concentrations of antibodies in certain fractions may result. Preliminary experiments based upon these ideas have been performed, and their success may be judged by a study of the data of Table V.

The small absolute amount of antibody content of normal human  $\gamma$ -globulin makes evaluation of such work difficult. Antibody is not destroyed by the action of the pepsin and in certain instances, especially in the case of the antiinfluenza A mouse protection assays, there seems to be an enhancement. Further work of this kind on  $\gamma$ -globulins of high antibody titer is contemplated.

The various globulin pastes were obtained from blood collected by the American Red Cross, after processing either at the Harvard Medical School pilot plant or in one of the cooperating pharmaceutical houses. We are indebted to Dr. Edwin J. Cohn and the members of the pilot plant staff at Boston for making these materials available to us. Furthermore, they have permitted us to use their pilot plant so that some experiments could be performed on a large scale.

In addition to the fact that they have carried out all of the antibody assays reported here, Dr. J. F. Enders and Miss J. C. Sullivan have given much valuable aid and encouragement in discussions related to the project. We are pleased to make record of indebtedness to them.

The clinical trials and preliminary guinea pig sensitization tests have been carried out by Dr. C. A. Janeway and his staff. Dr. Janeway's unfailing interest and inspiration and the care with which his share of the work was done have added much to the research.

We wish to thank Mr. E. M. Hanson, Mr. L. J. Gosting, and Mr. R. A. Alberty of this Laboratory for valuable technical assistance. It has been a most substantial contribution.

#### SUMMARY

By making use of a peptic digestion step and certain minor modifications, it has been shown to be possible to develop a process for the extraction of relatively pure  $\gamma$ -globulin from human protein residues containing  $\beta$ -globulins as well. The conditions for the large scale extraction have been set down in detail.

The greatest usefulness of the work probably will come in the hydrolytic cleavage of human  $\gamma$ -globulin antibody preparations in order to effect further concentration of the antibodies and to modify their absorption rate and distribution in the body. Representative immunological assay data demonstrate that antibody activities are not destroyed under the conditions which have been employed.

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# BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

## III. RECOVERY OF $\gamma$ -GLOBULIN FROM HUMAN BLOOD PROTEIN MIXTURES\*

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The recovery of  $\gamma$ -globulin from human blood globulin precipitates in high yield and without contamination by  $\beta$ -globulins is desirable because many of the antibodies are associated with the  $\gamma$ -globulins. The  $\gamma$ -globulins are concentrated in the plasma Fraction II + III of Cohn *et al.* (1). By-products which remain after part of the  $\gamma$ -globulin (Fraction II) has been recovered from Fraction II + III often contain substantial amounts of the valuable antibody material. The problem of increasing the antibody yield by the removal of further quantities of purified  $\gamma$ -globulin from such by-products has been approached by a method which involves a peptic digestion step (2). However, since the recovery of as much  $\gamma$ -globulin as possible is highly desirable and the operations may be carried out on a large scale, the attempt has been made to improve the theory and practice of the treatment of Fraction II + III and to simplify the process so that yields of  $\gamma$ -globulin will increase in Fraction II with correspondingly less of this substance remaining in residue materials. Granting success in this, it should be possible to treat a mixture of these  $\beta$ - and  $\gamma$ -globulins, in particular the Cohn Fraction II + III, so as to achieve a more efficient separation. A significant step in reaching this goal was taken when we observed that the effective separation of  $\gamma$ -globulin from the  $\beta$ -globulins requires a system of low ionic strength (about 0.010 instead of 0.040) as compared to that which had been in industrial use for this purpose up to the year 1945. (An earlier Harvard Medical School Method 6 designed for another purpose involves the use of a system of ionic strength 0.01, but the other conditions are radically different (3). By this method, there is obtained an isoagglutinin fraction, but no other fractions of clinical usefulness are separated.) Since the low ionic strength step has found its way into all subsequent directions for the extraction on the commercial scale of  $\gamma$ -globulin from pastes containing the  $\beta$ -globulins

\* This work has been carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin. The subject matter of this report has been used in Invention Disclosure 3607 of the Office of Scientific Research and Development (1945).

as well, and since the yields of antibody material have been substantially increased thereby, it is believed to be of interest to present the results of our work which have led to this development.

#### EXPERIMENTAL

The two proteins Fractions II + III and III-1 (from Harvard Medical School Method 3C) resulting originally as by-products of the commercial manufacture of human serum albumin were the source materials for the experiments to study the separation and recovery of the  $\gamma$ -globulin. In Figs. 1 and 2 are shown the Tiselius electrophoretic patterns of typical

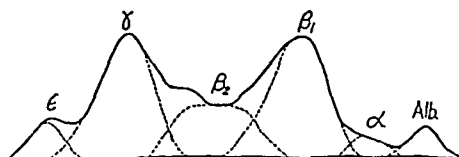


FIG. 1. Electrophoretic diagram for typical Fraction II + III. Barbiturate-citrate buffer at pH 8.6, potential gradient 10.2 volts per cm., time 7440 seconds.



FIG. 2. Electrophoretic diagram for typical Fraction III-1. Barbiturate-citrate buffer at pH 8.6, potential gradient 10.2 volts per cm., time 8100 seconds.

Fractions II + III and III-1, as obtained by means of the diagonal knife edge schlieren method. The electrophoreses were carried out at pH 8.6, with a diethyl barbiturate-citrate buffer of 0.088 ionic strength.<sup>1</sup> The average analytical results of typical experiments with standard Fraction III-1 (from Method 3C) and Fraction II + III pastes are shown in Table I.

These pastes were suspended in distilled water in the presence of ice by means of a Waring blender. The pH was then adjusted to 4.0 to 5.1 and the suspension was stirred for varying periods of time. This stage of the experiments will be referred to hereafter as the "suspension" stage. After suspension, the solutions were adjusted to various conditions of ionic strength, pH, and ethanol concentration in order to determine conditions for effective solution of the  $\gamma$ -globulin with simultaneous precipitation of

<sup>1</sup> 20 liters of this buffer may be prepared by bringing a solution of 184.2 gm. of diethyl barbiturate and 45.0 gm. of sodium citrate dihydrate to pH 8.6 with a sodium hydroxide solution and diluting it to 20 liters with conductivity water.

the  $\beta$ -globulins. This is the actual "fractionation" step. Since pH 5.2, ionic strength 0.029, and 15 per cent ethanol had been used in the fractionation step for the peptic digestion experiments, these conditions served as a guide for initial experiments. In turn, and except for the ionic strength, these conditions for the separation of  $\gamma$ -globulin from  $\beta$ -globulin were substantially those employed by the Department of Physical Chemistry of the Harvard Medical School. In all alcohol precipitations, the temperature was kept as close as possible to the freezing point of the solution. The proteins which were insoluble under these conditions were removed in a Sharples refrigerated laboratory centrifuge. The pH of the supernatant solution was then adjusted to 6.0 to 7.2 with sodium bicarbonate and the ethanol concentration brought to 25 to 30 per cent by volume with 95 per cent ethanol. The precipitated proteins, consisting largely of  $\gamma$ -globulin, were removed by centrifugation, suspended in cold distilled

TABLE I  
*Electrophoretic Analyses of Globulin Pastes*

Fraction No.	Electrophoresis analysis					
	$\gamma$ -Globulin	$\beta_2$ -Globulin	$\beta_1$ -Globulin	$\alpha_2$ -Globulin	$\alpha_1$ -Globulin	Albumin
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
II + III	37	20	36	2	1	4
III-1	27	14	42	11	4	2

water, frozen, and then dried *in vacuo*. The weight and electrophoretic analysis of the dried products were obtained and served for the evaluation of any given experiment.

Identical preliminary conditions were used in some of the experiments. In such cases sufficient starting material was taken to provide for several aliquots at the point where diverging conditions were introduced. This made for less tedious procedures and greater duplicability of conditions.

The ionic strength at the pH of the fractionation was calculated from the valence of the ions present and their concentration after the alcohol addition. Concentrations of the ions in the systems used were estimated from the aqueous dissociation constants of the corresponding acids at 25°. It was assumed that this procedure was applicable to alcoholic solutions at low temperatures. All pH determinations at the fractionation stages were made with a Beckman pH meter prior to alcohol addition and after warming the sample to 25°.

### Results

The object of our experiments was to discover a set of experimental conditions by which the antibody-rich  $\gamma$ -globulins of Fraction II + III

pastes could be effectively removed. The most readily available material with which to study the effects of variations in ionic strength, pH, and ethanol concentration in the recovery of these  $\gamma$ -globulins was the waste globulin by-product, Fraction III-1, which was obtained as a residue after the large scale treatment by the Harvard Medical School Method 3C of Fraction II + III for the production of human serum  $\gamma$ -globulin. It is to be emphasized that these Fractions III-1 are now systems of historical interest only, but, since they did serve so well in the establishment of more effective conditions for the removal and recovery of  $\gamma$ -globulin, we shall describe a number of our experiments with them. It is to be understood that the term Fraction III-1, as used without further qualification in subsequent paragraphs, always refers to a globulin residue obtained by the now obsolete Method 3C.

TABLE II  
*Preliminary Fractionation Experiments with Fraction III-1 Pastes*

Experiment No.	Suspension		Fractionation with 15 per cent ethanol		pH of pptn. with 30 per cent ethanol	Yield	Electrophoretic analysis			
	pH	Time	pH	$\frac{r}{2}$			$\gamma$ -Globulin	$\beta_2$ -Globulin	$\beta_1$ -Globulin	Albumin
		hrs.				gm.	per cent	per cent	per cent	per cent
A-2	5.15	0	5.15	0.029	6.7	11.4	88	3	1	9
A-6	4.0	14	5.16	0.015	6.3	11.8	89	3	1	9
A-8	4.0	18	5.15	0.029	6.3	11.9	84	3	2	11
A-9	4.0	12	5.09	0.013	6.9	15.0	87	8	1	4

In preliminary experiments, the adjustment to desired pH conditions during the suspension and fractionation stages was made with 0.05 N HCl and 0.05 N NaOH, and NaCl was used to raise the ionic strength to the desired level. Ethanol additions for the fractionation steps were made with 53.3 per cent ethanol, 95 per cent ethanol being used for the precipitation step. The results of typical preliminary experiments with 200 gm. portions of Fraction III-1 pastes containing approximately 22 to 25 gm. of  $\gamma$ -globulin in admixture with other proteins together with the conditions used during the various stages of each experiment are shown in Table II. The volume at the fractionation stage and after the addition of alcohol was 5.5 liters for each 200 gm. of Fraction III-1 paste used.

The conditions shown in Table II were satisfactory for a fair separation of  $\gamma$ -globulin from the  $\beta$ -globulins in Fraction III-1 pastes. Since the conditions for the fractionation stage were of paramount importance in influencing the yield of  $\gamma$ -globulin, the effect of changes in pH, ionic

strength, and ethanol concentration was next studied closely. The use of dilute HCl and NaOH for the adjustment of pH conditions was soon abandoned in favor of less drastic reagents. Dilute acetic acid (0.05 M) or dilute pH 4.0 acetate-phosphate solution<sup>2</sup> was used to lower the pH, while sodium acetate or disodium phosphate solutions were used to adjust the pH to higher values.

In a series of experiments carried out with these reagents, the effect of ionic strength change in the fractionation stage was determined. Other variables were maintained as constant as possible. The results obtained

TABLE III  
*Effect of Ionic Strength, pH Variation, and Ethanol Concentration*

Experiment No.	Suspension		Fractionation			pH of pptn. with 25 per cent ethanol	Yield	Electrophoretic analysis			
	pH	Time	pH	Ethanol	$\frac{\Gamma}{2}$			$\gamma$ -Glo- bulin	$\beta_2$ -Glo- bulin	$\beta_1$ -Glo- bulin	Albu- min
Effect of ionic strength											
		hrs.		per cent			gm.	per cent	per cent	per cent	per cent
A-20A	5.1	14	5.10	17	0.01	7.01	17.0	89	5	4	2
A-20B	5.1	14	5.09	17	0.03	7.03	13.8	89	4	1	6
A-20C	5.1	14	5.08	17	0.05	7.03	10.5	92	5	0	3
Effect of pH variation											
A-16A	4.92	16	4.99	17	0.008	7.03	20.7	74	15	6	5
A-16B	4.92	16	5.09	17	0.0095	7.02	14.4	94	3	0	3
A-16C	4.92	16	5.20	17	0.011	6.92	11.4	95	2	0	3
Effect of ethanol concentration											
A-12A	4.15	12	5.08	13	0.012	6.75	18.5	86	8	2	4
A-12B	4.15	12	5.08	15	0.010	7.13	15.5	91	4	1	4
A-12C	4.15	12	5.08	17	0.010	7.03	14.6	97	2	0	1

with 200 gm. portions of Fraction III-1 paste indicate that highest yields, without much loss in electrophoretic purity, may best be obtained at an ionic strength of approximately 0.01, as is shown in Table III.

The use of still lower salt concentrations was not investigated in this series, since previous experiments had shown that ionic strengths appreciably below 0.01 at pH 5.1 and 15 to 17 per cent ethanol failed to precipitate a large part of the  $\beta_2$ -globulins present in the starting material. Thus, in one experiment in which the fractionation was carried out at pH 5.01,

<sup>2</sup> This pH 4.0 solution is prepared by mixing equal volumes of 0.043 M acetic acid and 0.007 M disodium phosphate.



17 per cent ethanol, and an ionic strength of 0.002, the final product contained 37 per cent of  $\beta_2$ -globulin and 57 per cent of  $\gamma$ -globulin.

Variations in pH at the fractionation stage were studied by using 0.05 M acetic acid and 0.05 M disodium phosphate to adjust the pH. In spite of the fact that phosphates have little or no buffering capacity at pH 5.1, their use in raising the pH from that of the suspension level seemed to give somewhat better results and higher purity of product as compared to acetate. Although the ionic strengths were not maintained constant and secondary effects may have occurred, the major changes seen in Table III have been shown by our experience to be due to pH variations in the fractionation step. The results in Table III show that suitable yields of  $\gamma$ -globulin with high purity are dependent on a pH of essentially 5.10 at an ionic strength of approximately 0.01. In these experiments 17 per cent ethanol was used. Again, 200 gm. of Fraction III-1 paste served as starting material. The large differences in yield brought about by a variation in pH of only 0.1 unit indicate the care which must be taken in making pH adjustments at this point.

The effect of a third variable, the concentration of ethanol, was likewise investigated. The pH adjustments were effected by the use of dilute acetic acid and disodium phosphate solutions. From Table III it can be seen that optimum results are obtained with 17 per cent ethanol when the ionic strength and pH are maintained at 0.01 and 5.10, respectively.

The effect of temperature was not studied as another variable, because the temperature at the fractionation stage was always kept as close as possible to the freezing point of the solution. In this way tendencies towards denaturation are minimized.

As a result of many experiments, not reported here in detail, it was found that certain variations in procedure introduced small deviations in the final result. Thus the suspension of Fraction III-1 pastes at pH values somewhat acid to pH 5.1 tended to increase the  $\gamma$ -globulin yields, while extension of the time of extraction from several hours to 16 or more hours appeared to give a slightly favorable effect. Use of pH values of 7.0 or above for the final 25 per cent ethanol precipitation of  $\gamma$ -globulin gave rise to a desired greater loss of albumin into the final supernatant solution.

### *Method*

The final conditions adopted for the separation and recovery of  $\gamma$ -globulin from pastes containing large amounts of  $\beta$ -globulin, Fraction II + III, are essentially those of Experiment A-12C of Table III. The details have been incorporated into a formal scheme which may be presented in the form of directions for procedure. These directions are given for the

treatment of 1 kilo quantities of Fraction II + III paste. Since the protein content and pH of Fraction II + III pastes may vary, corrections should be made for any pH deviations from the values as given. The ionic strength at the fractionation stage is calculated from the volume including the alcohol added to give 17 per cent concentration and should always be maintained at 0.010 to 0.014 for the separation.

### Fraction II + III

Each kilo of this precipitate is suspended in 10 liters of 0° pyrogen-free water and 4850 ml. of a dilute pH 4.0 solution are added in order to bring the pH of the system to  $4.8 \pm 0.05$ .<sup>3</sup> After the system has been slowly

TABLE IV  
Preparation of  $\gamma$ -Globulin from Fraction II + III Paste

Experiment No.	Suspension		Fractionation with 17 per cent ethanol		pH of pptn. with 25 per cent ethanol	Yield	Electrophoretic analysis			
	pH	Time	pH	$\frac{r}{2}$			$\gamma$ -Globulin	$\beta_1$ -Globulin	$\beta_2$ -Globulin	Albumin
		hrs.				gm.	per cent	per cent	per cent	per cent
A-6A	5.11	15	5.20	0.014	6.85	16.7	97	2	0	1
A-7	4.80	10	5.09	0.012	6.85	18.8	97	2	0	1
A-8C	4.81	12	5.09	0.015	6.88	17.0	97	2	0	1
A-8D	4.81	24	5.09	0.015	6.92	15.2	98	1	0	1
A-9B	4.76	0.3	5.09	0.016	6.94	15.1	97	2	0	1
A-11	4.79	18	5.09	0.012	6.85	18.9	97	1	1	1

stirred for a period of hours at 0°, a buffer mixture of 2640 ml. of 0.050 M  $\text{Na}_2\text{HPO}_4$  and 2260 ml. of 0.050 N acetic acid is added, again at 0°, to bring the pH to  $5.1 \pm 0.04$ .

The solution is now diluted to a volume of 25.0 liters with pyrogen-free water, at 0°, and the ethanol concentration is brought to 17 per cent by the cautious addition of 11.25 liters of 53.3 per cent alcohol. The ionic strength is 0.012 at this point. During this addition, the temperature is kept as low as possible, without ice formation; it should be  $-6.5^\circ$  when 17 per cent concentration of alcohol is reached. After being stirred for 1 hour at this temperature, the precipitate is removed by centrifugation, at  $-6.5^\circ$ .

The effluent is clarified by filtration through a thin pad of a suitable filter aid. After the pH of the filtrate is adjusted to  $7.0 \pm 0.1$  by the addition of 0.50 M  $\text{NaHCO}_3$  (about 900 ml. are required), the alcohol con-

<sup>3</sup> This solution is prepared by adding 680 ml. of 0.050 M  $\text{Na}_2\text{HPO}_4$  to 4170 ml. of 0.050 N acetic acid.

centration is increased to 25 per cent by the slow addition of 95 per cent ethanol which has been cooled to  $-5^{\circ}$  or colder. The precipitate is removed by centrifugation at a temperature not higher than  $-5^{\circ}$  and is dried from the frozen state in the usual manner.

The material thus secured has a purity of about 95 per cent with respect to  $\gamma$ -globulin and is obtained in a yield of approximately 75 to 80 per cent. Data of some typical experiments with 200 gm. portions of Fraction II + III pastes containing approximately 22 to 26 gm. of  $\gamma$ -globulin are shown in Table IV.

A typical electrophoretic pattern in diethyl barbiturate-citrate buffer at pH 8.6 is shown in Fig. 3.

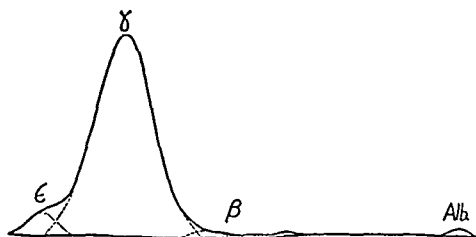


FIG. 3. Electrophoretic diagram for product recovered in Experiment A-9B on Fraction II + III. Barbiturate-citrate buffer at pH 8.6, potential gradient 10.3 volts per cm., time 7320 seconds.

#### DISCUSSION

The recovery of  $\gamma$ -globulin from human blood protein pastes, designated as Fraction II + III, and prepared by the low temperature ethanol precipitation methods of Cohn *et al.* has been investigated and substantial increases in yield without sacrifice of purity have been secured. Significant decreases in the amount of  $\gamma$ -globulin which is "salted-out" with the  $\beta$ -globulin residue take place at the fractionation step when relatively low ionic strengths (0.010 as compared to 0.040 in the prior commercial procedures) are used. For high yields of  $\gamma$ -globulin, pH and ethanol concentration must be accurately controlled.

The standard Fraction II, designated as Fraction II-1,2 by Oncley *et al.* (3), represents the more soluble part of the  $\gamma$ -globulin. Such material has been used clinically in the treatment of both measles (4, 5) and epidemic jaundice (6, 7). The additional  $\gamma$ -globulin recovered by the application of our conditions, the Fraction II-3 of Oncley *et al.* (3), has not been widely used in clinical studies, but the immunological titers of the antibodies are almost identical with those reported by Enders (8) for the standard material. In the case of influenza A (mouse protection) and typhoid "O" antibodies,

the titers for our fraction seem to be definitely enhanced. There is no reason to anticipate that the new globulin fraction will be found to be less potent than the old on clinical study, because the  $\gamma$ -globulin recovered from Fraction III-1 residues by the pepsin digestion process (2), which corresponds to Fraction II-3, has already been subjected to trial and found to give results which are consistent with those obtained by using the standard Fraction II-1,2  $\gamma$ -globulin.

Concentration of antibody from certain plasmas from convalescents has received added impetus now that it has been shown to be possible to recover some three-quarters of the total  $\gamma$ -globulin from Fraction II + III pastes. In this case, it will be evident that optimum yields are highly desirable, since relatively small amounts of such plasmas can be obtained for processing.

Intravenous injection in cats of solutions of the additional  $\gamma$ -globulin recovered from Fraction III-1 as well as of  $\gamma$ -globulin from Fraction II + III has disclosed that the majority of the samples show rather marked hypotensive properties. In this respect they are neither better nor worse than the standard material. It is true, however, that when the suspension step is carried out at reactions in the range pH 3.5 to 4.5 (prior to fractionation) samples which are less depressive are usually obtained.

From the point of view of their physicochemical characterization, standard and additional  $\gamma$ -globulins are similar; sedimentation and electrophoretic diagrams, heat stability, and behavior are alike. Presumably our Fraction II-3 contains more euglobulin than does the standard Fraction II-1,2, because what has been accomplished is the recovery of some of the less soluble  $\gamma$ -globulin.

We are greatly indebted to Dr. J. F. Enders and to Miss J. C. Sullivan of the Department of Bacteriology and Immunology, Harvard Medical School, for their interest and for the performance of all immunological assays.

The raw materials, by-product pastes containing  $\beta$ - and  $\gamma$ -globulins, were obtained through the generous cooperation of Dr. F. A. Eberly and his associates at The Upjohn Company, Kalamazoo, Michigan.

#### SUMMARY

In all but the latest commercial methods for the preparation of human  $\gamma$ -globulin, the yields have not been in excess of 50 per cent. The remaining  $\gamma$ -globulin, together with a corresponding quantity of the antibody, was precipitated in a residue together with the  $\beta$ -globulins. A step in providing a substantial increase in the  $\gamma$ -globulin yield was taken when it was discovered by us that effective separation of  $\gamma$ - from  $\beta$ -globulins requires a

system of much lower ionic strength than had been in use in large scale commercial operation. In the present work, fractionation conditions of pH 5.1, ionic strength 0.01, alcohol concentration 17 per cent, and temperature  $-6^{\circ}$  are used. When the new experimental conditions are applied directly to the main  $\beta$ - and  $\gamma$ -globulin fraction (Fraction II + III), more than three-quarters of the total  $\gamma$ -globulin may be recovered in a single step, and without sacrifice in purity.

This development is an important item leading to practical and economical fractionation methods for the removal of antibody from plasmas or sera of convalescents and hyperimmune individuals.

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# THE BIOCHEMISTRY OF THE MALARIA PARASITE

## V. MECHANISM OF PYRUVATE OXIDATION IN THE MALARIA PARASITE\*

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Previous work on the carbohydrate metabolism of malaria parasites (1, 2) has indicated that utilization of glucose by these organisms involves the initial formation of lactic acid and the subsequent oxidation of this substance to carbon dioxide and water. The rate of formation of lactate exceeds the rate of oxidation, with the result that lactate accumulates even under aerobic conditions. The oxygen consumption of parasitized erythrocytes is about the same with pyruvate or lactate as substrate as with glucose. Under anaerobic conditions, lactate is apparently the only product of glucose utilization. An investigation of the mechanism of glycolysis occurring in cell-free extracts of the malaria parasite (3) has indicated that this process involves the same reactions and the same phosphorylated intermediates as are found in yeast and muscle.

The similarity of the reactions in the parasites to those occurring in organisms which are known to oxidize glucose through pyruvate as an intermediate suggested that glucose is oxidized in the same manner by the malaria parasites. The most generally accepted mechanism for the oxidation of pyruvate is the tricarboxylic acid cycle of Krebs (4). However, the reports of previous workers that succinate, fumarate, malate, and citrate were not oxidized by the malaria parasite (5, 6) and that malonate did not inhibit carbohydrate oxidation (1) seemed to exclude this mechanism in these organisms.

Since the earlier experiments were carried out with suspensions of intact parasitized erythrocytes, it appeared possible that the failure to obtain any effect with the polycarboxylic acids was due to the impermeability of the erythrocyte membrane to these highly polar substances or to some other property of the intact erythrocyte. Accordingly, we have studied the metabolism of the parasite *Plasmodium gallinaceum*, both in intact erythrocytes and when freed from the erythrocytes by the use of hemolytic

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serum. We have found that in both preparations pyruvate is oxidized to carbon dioxide and water by a cycle like that proposed by Krebs for muscle tissue. In free parasites, the oxidation of pyruvate to acetate is an additional important pathway of metabolism. Evidence has been obtained that the oxidation of glucose and lactate proceeds over the same pathways as does the oxidation of pyruvate.

## EXPERIMENTAL

### *Materials*

The citric, fumaric, *l*-malic, malonic, and succinic acids employed were recrystallized commercial products. Solutions of glucose and sodium acetate were prepared from the analytically pure anhydrous compounds. Crystalline sodium pyruvate was prepared by the method of Lipmann (7). It was 100 per cent pure, as determined by decarboxylation with yeast carboxylase and by oxidation with acid permanganate. Crystalline lithium lactate was prepared according to the directions of Friedemann and Graeser (8). Oxalacetic acid was obtained by the hydrolysis of commercial sodium diethyl oxalacetate (9).  $\alpha$ -Ketoglutaric acid was synthesized by the method of Neuberg and Ringer (10). *cis*-Aconitic acid was made by the procedure of Malachowski *et al.* (11) from *trans*-aconitic acid synthesized according to Bruce (12). All acids were neutralized to pH 7.4 with NaOH before use.

Diphosphopyridine nucleotide was prepared by the method of Williamson and Green (13). Triphosphopyridine nucleotide was obtained by the method of Warburg, Christian, and Grieser (14), as modified by Altman (15). Adenosine triphosphate was isolated from the skeletal muscle of rabbit by the procedure described by Needham (16). Flavin-adenine-dinucleotide was prepared according to Warburg and Christian (14). Riboflavin phosphate was synthesized by the method of Kuhn and Rudy (17). Thiamine, diphosphothiamine, nicotinic acid, nicotinamide, riboflavin, calcium pantothenate, *p*-aminobenzoic acid, pyridoxine, inositol, and biotin were obtained in pure form from commercial sources.

### *Analytical Methods*

Measurements of *gas exchange* were made with a Barcroft-Warburg manometric apparatus, according to the methods described by Dixon (18). When respiratory quotients were measured, initial and final bound carbon dioxide was determined by tipping in acid from the side arm, and two vessels were carried through the experimental period, one with KOH in the center cup for measurement of oxygen consumption and a second without KOH for measurement of the sum of carbon dioxide production and oxygen consumption.

*Glucose* was determined by the method of Nelson (19). *Succinate* was measured by the method of Szent-Györgyi and Gözsy (20), as modified by Krebs and Eggleston (21, 22). *Malate* was determined by an unpublished method from this laboratory, based on the fluorescence obtained by heating malate with  $\beta$ -naphthol in concentrated sulfuric acid (23).

*Lactate* was determined by the method of Barker and Summerson (24), except that 9.0 cc. of concentrated sulfuric acid were used instead of 6.0 cc. in order to decrease the interference from pyruvate. It was noted, in agreement with Miller and Muntz (25), that when samples were not carried through a copper-lime precipitation the interference caused by pyruvate decreased as the heating time was increased from 5 to 15 minutes, but the absolute color intensity obtained with a given quantity of lactate also decreased markedly with longer heating. When a copper-lime precipitation was employed, the interference by pyruvate was less than without copper-lime precipitation and was independent of the heating time. Either with or without copper-lime precipitation, the use of larger quantities of sulfuric acid decreased the interference by pyruvate. Under the conditions used (copper-lime filtrates, 9.0 cc. of sulfuric acid, 5 minutes heating time), pyruvate gave 2 per cent of the color obtained with an equimolar quantity of lactate.

*Pyruvate* was determined by the procedure of Friedemann and Haugen (26). The 2,4-dinitrophenylhydrazine solution was allowed to react with the samples for 5 minutes at room temperature, and the reaction mixture was extracted with 3.0 cc. of toluene. It was found convenient to carry out the reaction and the extractions in glass-stoppered, conical tipped centrifuge tubes of about 30 cc. capacity.<sup>1</sup> After the hydrazone was extracted by shaking with toluene for 30 seconds, the aqueous layer was removed with a long capillary pipette connected by means of glass tubing bent in the shape of an inverted U to a 20 cc. syringe with a greased barrel.

*Acetate* was determined by the following procedure, based in part upon the methods of Leloir and Muñoz (27) and Long (28). Zinc filtrates were prepared by adding 5.0 cc. each of 5 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.3 N NaOH to each 3.0 cc. sample. The mixture was diluted to 27 cc. and centrifuged. If glucose was present in the sample, a 20 cc. aliquot of the zinc filtrate was diluted to 26 cc. and treated with 2 cc. of 20 per cent  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 2 gm. of solid  $\text{Ca}(\text{OH})_2$ . After standing at room temperature for at least 2 hours, the mixture was centrifuged.

To a 20 cc. aliquot of the zinc or copper-lime filtrate were added 3.0 cc. of a distillation reagent similar to that used by Long (28). The reagent contained 1300 mg. of 2,4-dinitrophenylhydrazine and 70 mg. of lithium

<sup>1</sup> Maizel-Gerson reaction vessels, from the Wilkens-Anderson Company, Chicago.



lactate in 100 cc. of 24 N  $\text{H}_2\text{SO}_4$  (200 micromoles of 2,4-dinitrophenylhydrazine and 20 micromoles of lactic acid in each 3.0 cc.). If a precipitate formed after 1 hour in the ice box, it was removed by filtration through a fritted Pyrex glass filter. The mixture of sample and distillation reagent was then washed into a 100 cc. round bottom flask with a standard taper joint. To the flask were added 10 gm. of anhydrous  $\text{Na}_2\text{SO}_4$  and three small carborundum chips to promote even boiling. The flask was attached to a modified Claisen head which formed an all-glass distillation apparatus. The straight neck of the Claisen head was provided with a standard taper joint to receive a dropping funnel, and the bent neck was extended as a short asbestos-wrapped Vigreux column. The column was connected through a Kjeldahl trap to a vertical condenser, and a 200 cc. round bottom flask was attached to the bottom of the condenser by a standard taper connection to serve as a receiver. The small flask was heated with the free flame of a micro burner, and, as distillation proceeded, the volume of liquid in the distilling flask was kept constant by addition of water from the dropping funnel. Distillation was interrupted when 100 cc. of distillate had been collected. The distillate was boiled for 10 seconds and titrated while hot with  $\text{CO}_2$ -free 0.01 N NaOH, with brom-thymol blue as indicator. A blank distillation was performed and its titration value subtracted from that of the other samples.

After treatment with the distillation reagent, samples containing as much as 100 micromoles of pyruvic acid could be distilled without perceptible interference from pyruvate. Virtanen and Pulkki (29) have found that as the concentration of lactic acid in a solution is increased the quantity of acid which distills off does not increase proportionally but reaches a low maximum value. On the basis of this observation, which we have confirmed, we eliminated interference from lactic acid by carrying out all distillations in the presence of an excess of lactic acid and correcting for the quantity distilling over by a blank titration. Interference from glucose was effectively prevented by the copper-lime precipitation. By the procedure outlined above, 90 to 95 per cent of added acetic acid could be recovered consistently from pure solutions or from filtrates. Agreement between duplicates was of the order of 2 to 5 per cent.

When it was desired to identify the volatile acids formed during an experiment, the combined distillates were exactly neutralized with a few mg. of solid  $\text{CaCO}_3$  and decolorized with charcoal to remove the indicator. The filtered solution was concentrated to 2 to 3 cc. volume at atmospheric pressure, filtered again, and used for Duclaux distillations or for color tests.

*Citrate* was determined by a method incorporating certain features of several previously published procedures. The colorimetric pentabromoacetone method of Perlman, Lardy, and Johnson (30) offers advantages in

convenience of manipulation, but in our experience the color produced is unstable, the duplication erratic, and the sensitivity low. In confirmation of Hunter and Leloir (31), the use of sodium nitrite to reduce the permanganate and particularly the use of ethylene glycol to stabilize the color were found to represent great improvements. When these modifications were applied to the method of Perlman, Lardy, and Johnson, the blanks showed a strong yellow color. We observed that this color could be avoided by destroying the excess nitrite with urea. In the present method, the recommendations of Krebs and Eggleston (32) as to acid concentration during the oxidation and bromination were followed.

Samples to be analyzed for citrate were deproteinized by the addition of an equal volume of 1 M trichloroacetic acid. An aliquot containing 0.5 to 5.0 micromoles of citrate (0.096 to 0.960 mg. of citric acid) in a volume not exceeding 7 cc. was placed in a graduated 15 cc. centrifuge tube along with 2.25 cc. of 15 N  $\text{H}_2\text{SO}_4$  and diluted to about 9 cc. The samples were heated 1 hour in a boiling water bath, cooled, treated with an excess of saturated bromine water (about 10 drops), and diluted to 9.0 cc. After 15 minutes at room temperature, the tubes were centrifuged. This procedure eliminated interference from acetone, acetoacetate, and oxalacetate.

Two 4.0 cc. aliquots of the acid-bromine-treated filtrate were transferred to conical tipped, glass-stoppered centrifuge tubes of the type used in the pyruvate determinations. A blank and three different standards, in a volume of 3.0 cc., were prepared, and 1.0 cc. of 15 N  $\text{H}_2\text{SO}_4$  was added to each. Each subsequent operation was carried out with the whole series of tubes before proceeding to the next step. To the samples were added 0.2 cc. of 1 M KBr and 0.8 cc. of 0.3 M  $\text{KMnO}_4$ . After 15 minutes at room temperature, the excess permanganate was reduced by the addition of 0.5 cc. of 1.5 M  $\text{NaNO}_2$ , with thorough mixing to insure complete reaction. Then 0.5 cc. of 2 M urea was added, with complete mixing and rinsing of the vessel walls. When more than 1 micromole of citrate was present in the aliquot analyzed, a faint white precipitate of pentabromoacetone was visible. The pentabromoacetone was extracted by shaking for 40 seconds with 10 cc. of acid-washed 60–70° petroleum ether (Skellysolve B). The aqueous layer was removed as completely as possible with a capillary pipette attached to a greased syringe. The petroleum ether was washed once by adding 3 cc. of water, rinsing the stopper and ground glass seat, shaking for 15 seconds, and again removing the aqueous layer. 5.0 cc. of sodium sulfide-ethylene glycol reagent were added to each of the tubes without mixing. The reagent was prepared by dissolving 5 gm. of wet crystalline sodium sulfide in 60 cc. of water, adding 40 cc. of ethylene glycol, and filtering. The tubes were shaken vigorously for 1 minute, and the contents were poured into 16 X 150 mm. Pyrex test-tubes calibrated for use

as colorimeter tubes. The samples were read in an Evelyn colorimeter, with a special sleeve to accommodate the tubes and filter No. 420.

A plot of the  $L$  values ( $2 - \log$  of per cent transmission) against the amount of citrate added gave a curve which was slightly concave upward for values up to 0.6 micromole of citrate and was linear for higher values. Duplicates agreed within 5 per cent, and the standard curve was reproducible within the same limits. The yellow color formed by a given amount of citrate was much more intense than that obtained by other methods and was fairly stable. Readings could be begun about 10 minutes after mixing the petroleum ether and sodium sulfide-ethylene glycol reagent in the first tube; in the next 15 minutes the color faded about 4 to 5 per cent at all levels of citrate. The range of the method could be extended to include larger amounts of citrate by doubling the volume of sodium sulfide-ethylene glycol reagent.

### *Parasite Preparations*

White Leghorn chickens infected with *Plasmodium gallinaceum*<sup>2</sup> served as the source of malaria parasites. Chickens weighing 1 to 2 kilos were inoculated intravenously with parasitized blood, and the peak of parasitemia was reached on the 3rd day after injection, with 60 to 90 per cent of the erythrocytes containing one or more parasites. At this time blood was drawn by heart puncture, with the addition of 0.05 volume of 10 per cent sodium citrate to prevent coagulation. From the citrated blood, two types of parasite preparations were made—parasitized erythrocytes and free parasites.

*Parasitized Erythrocytes*—The cells were separated from the citrated blood by centrifuging for 5 minutes at 500*g* (1600 R.P.M., head radius 18.5 cm.) and were washed three times by suspending in 10 volumes of cold calcium-free phosphate-saline, pH 7.4 (22), and centrifuging again. The washed cells were suspended in phosphate-saline to a hematocrit value of about 30 per cent.

*Free Parasites*—A preliminary study of various procedures for laking the erythrocytes indicated that specific hemolytic serum more consistently yielded free parasites with a rapid oxygen consumption than did other agents tried (water, digitonin, saponin, synthetic detergents). The serum was prepared by injecting rabbits intravenously at intervals of 3 or 4 days with washed erythrocytes from normal chickens, in doses increasing from 0.1 to 1.0 cc. of cells over a period of 3 weeks. The serum obtained in this way completely hemolyzed an equal volume of washed erythrocytes in 5 to 10 minutes under the conditions described below.

To prepare free parasites, parasitized erythrocytes obtained from citrated

<sup>2</sup> Strain 8A, as designated by the Committee on Terminology of Strains of Avian Malaria of the American Society of Parasitologists.

blood were washed twice with phosphate-saline containing 0.1 per cent glucose and suspended to a hematocrit value of about 15 per cent. The suspension was incubated for 15 minutes at 40° with a volume of hemolytic serum equal to the volume of the erythrocytes and a volume of guinea pig serum complement<sup>3</sup> equal to one-tenth that of the erythrocytes. The mixture was shaken frequently to resuspend the agglutinated cells. The solid material was separated by centrifuging for 5 minutes at 900*g* (2100 R.P.M.) and was washed three times by suspending in 10 volumes of cold phosphate-saline. Phosphate-saline was finally added to give a suspension containing about 60 per cent solids.

The free parasite material was dark gray-brown in color. Smears treated with Giemsa stain showed the parasites to be completely free from the erythrocytes. The parasites were slightly contracted in appearance but the nuclei and cytoplasm stained normally. Lymphocytes and erythrocyte nuclei were also present. The free parasites were capable of causing fatal infections when injected into chicks, although the infectivity was much less than that of intact parasitized erythrocytes, as indicated by a prolonged induction period.

The content of solids in the parasite preparations estimated by centrifuging the suspensions for 5 minutes at 1700 *g* (2800 R.P.M.) and most of the data of this paper are expressed as change per cc. of solids determined in this manner. When both parasitized erythrocytes and free parasites were prepared from the same samples of blood, the volume of free parasites derived from a given volume of parasitized erythrocytes could be estimated. Generally the parasitized erythrocytes yielded about an equal volume of free parasites.

To avoid possible osmotic effects on the intact cells, it was necessary to control the ionic composition of the medium in which the samples were incubated. Whenever possible, all added solutions were adjusted to a cation concentration of 0.150 *M*. Any necessary adjustment in the final concentration of cations in the reaction mixture was made by addition of water or concentrated solutions of NaCl. In each experiment, the cation composition of all samples was the same. It was found that variation of the concentration of cations from 0.14 to 0.18 *M* had no marked effect upon the metabolism of the parasites.

## Results

### *General Metabolic Properties of Parasite Preparations*

#### *Respiration of Parasite Preparations in Absence of Added Substrates—*

Both parasitized erythrocytes and free parasites respire at an appreciable rate in the absence of added substrates but the substance or substances

<sup>3</sup> Lyovac complement, from Sharp and Dohme, Inc.

being oxidized under these conditions have not been identified. Analyses indicate that glucose, pyruvate, malate, and citrate are absent. Lactate and reducing substances liberated by acid hydrolysis are present only in small amounts and do not undergo significant change. The changes in non-protein nitrogen fractions of parasitized erythrocytes are discussed in the following paper (33). There is evidence that the material being oxidized is in about the same oxidative state as carbohydrate, since the respiratory quotient of the blank respiration in free parasites is about 0.9. Furthermore, the blank respiration of both parasitized erythrocytes and free parasites is strongly inhibited by malonate, with the accumulation of small amounts of succinate. This suggests that the blank oxidation involves the tricarboxylic acid cycle. In a similar manner, minced muscle tissue in the absence of added substrates shows a malonate-sensitive respiration of high respiratory quotient, which cannot be accounted for by changes in known carbohydrate material (34).

It is not known to what extent oxidation of this unknown substrate is displaced when other substrates are added, but in calculation and discussion it is assumed that rapidly oxidized substrates such as pyruvate completely replace the unknown substance.

*Effect of Cofactors on Metabolic Properties of Parasite Preparations*—The rate of pyruvate oxidation by parasitized erythrocytes is not affected by added cofactors, but the rate of oxidation by free parasites is increased by the addition of dicarboxylic acids and other water-soluble cofactors. The catalytic effects of dicarboxylic acids such as succinate or malate are discussed in a later section. In the presence of dicarboxylic acids, addition of diphosphothiamine, thiamine, diphosphopyridine nucleotide, or triphosphopyridine nucleotide causes a further acceleration of both oxygen consumption and pyruvate utilization, to an extent dependent upon the concentration added (Table I). Adenosine triphosphate usually has a similar effect, but its action is variable. Low concentrations of manganous ions are stimulatory, but higher concentrations inhibit pyruvate oxidation. A mixture of these known cofactors, such as to give the final concentrations indicated in the last section of Table I, was usually added to samples of free parasites in which the metabolism of pyruvate was being studied, and in later tables is called simply "cofactor mixture."

The following water-soluble vitamins and their derivatives have no effect upon pyruvate oxidation in free parasites: riboflavin, riboflavin phosphate, flavin-adenine-dinucleotide, nicotinic acid, nicotinamide, *p*-aminobenzoic acid, pantothenic acid, pyridoxine, biotin, and inositol. Spermine and spermidine, which antagonize the bacteriostatic effect of atabrine (35), are without influence. Glutathione is also inactive.

Boiled extracts of yeast and of chicken tissues were prepared by suspend-

ing the material in one part of phosphate-saline, heating 10 minutes in a boiling water bath, and removing the solids by centrifugation. Such extracts prepared from normal and parasitized chicken erythrocytes, chicken liver, and chicken breast muscle accelerate the oxidation of pyruvate by free parasites, but not to a greater extent than can be achieved

TABLE I

*Effect of Cofactors on Pyruvate Oxidation by Free Parasites*

The samples contained 1.2 cc. of free parasites, 0.01 M pyruvate, 0.00333 M malate, and the cofactors indicated below, in a volume of 3.0 cc. Gas phase air, temperature 40°, time 60 minutes.

Cofactor	Concentration	Increase in	
		Oxygen used	Pyruvate used
	M	per cent	per cent
Thiamine.....	0.00001	3	6
	0.00005	6	4
	0.00025	6	5
Diphosphothiamine..	0.00005	4	8
	0.00025	9	10
Diphosphopyridine nucleotide.....	0.00001	1	4
	0.00005	8	8
	0.00025	16	16
Triphosphopyridine “.....	0.00001	8	6
	0.00005	14	10
	0.00025	22	18
Adenosine triphosphate.....	0.0001	5	6
	0.0005	3	11
	0.0025	-2	13
Manganous chloride.....	0.0002	10	11
	0.0005	-11	9
Cofactor mixture.....		23	12
Diphosphothiamine.....	0.00005		
Diphosphopyridine nucleotide.....	0.00005		
Triphosphopyridine nucleotide.....	0.00005		
Adenosine triphosphate.....	0.0005		
Manganous chloride.....	0.0001		

with the cofactor mixture of Table I. On the other hand, extracts of Fleischmann's bakers' yeast have a much greater effect than does the cofactor mixture, increasing oxygen consumption as much as 100 per cent. This effect was traced to the surprisingly high succinic acid content of the yeast (at least 0.2 per cent of the dry weight). The other extracts do not contain significant quantities of ether-extractable acids which stimulate the respiration of the parasites. These results with crude extracts render

unlikely the existence of any unknown cofactor which can greatly accelerate the rate of pyruvate oxidation in free parasites.

*Relative Metabolic Activity of Parasitized Erythrocytes and Free Parasites*—In order to compare the respiratory activity of free parasites with that of parasitized erythrocytes,<sup>4</sup> the oxidation of a variety of substrates by these two types of parasite preparations was investigated, and the results are given in Table II. In general, the free parasites oxidize these substances at about half the rate found with an equivalent quantity of parasite material within the intact erythrocyte. It is noteworthy that succinate is utilized in significant amounts by both free parasites and parasitized

TABLE II

*Oxidation of Various Substrates by Parasitized Erythrocytes and Free Parasites*

The samples contained parasitized erythrocytes equivalent to 0.6 cc. of free parasites (carried through the procedure for the preparation of free parasites, except for the addition of antiserum) or 1.1 cc. of free parasites (both preparations from the same sample of blood), cofactor mixture, and the substrates indicated below in 0.01 M concentration (with 0.00033 M malate in the case of glucose, lactate, and pyruvate), in a volume of 3.0 cc. Gas phase air, temperature 40°, time 60 minutes. The experimental values are expressed in micromoles per hour per cc. of free parasites.

Substrate added	Oxygen consumed			Substrate oxidized		
	Parasitized erythrocytes	Free parasites	Free Erythrocytic	Parasitized erythrocytes	Free parasites	Free Erythrocytic
	micromoles	micromoles		micromoles	micromoles	
None.....	21.1	6.5	0.31			
Glucose.....	48.1	14.6	0.30	23.0	11.9	0.52
Lactate.....	61.9	28.8	0.47	24.1	8.7	0.36
Pyruvate.....	58.8	27.0	0.46	29.8	24.0	0.80
Succinate.....	24.8	12.9	0.52	24.1	13.0	0.54
Malate.....	23.8	16.2	0.68	4.1	18.0	4.39

erythrocytes, while malate is only slowly used by parasitized erythrocytes. The respiratory rate of parasitized erythrocytes in the presence of adequate substrate is linear for 3 to 4 hours, but the respiratory rate of free parasites declines considerably after the first hour.

The data of Table III indicate that the glycolytic activity of free parasites is unaltered, but that they are unable to oxidize the lactate formed from glucose as rapidly as parasitized erythrocytes. The high acidity resulting from the accumulation of such large amounts of free lactic acid (more than

<sup>4</sup> It has been reported previously (2) that suspensions of washed parasitized erythrocytes in calcium-free phosphate-saline respire at the same rate as do comparable samples of parasitized whole blood.

0.01 N) is undoubtedly responsible for the low oxygen consumption of free parasites with glucose as substrate.

The respiratory activity of preparations made from normal chicken blood was determined under conditions identical with those used for parasite preparations, and these data are shown in Table IV. The normal preparations corresponding to free parasites contained only erythrocyte nuclei

TABLE III

*Utilization of Glucose by Parasitized Erythrocytes and Free Parasites*

These data were obtained from the experiments described in Table II. The experimental values are expressed in micromoles per hour per cc. of free parasites.

Parasite preparation	Glucose oxidized	Lactate formed	Total glucose utilized	Glucose utilized, Free Erythrocytic
	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	
Parasitized erythrocytes. . . . .	23.0	1.2	23.6	
Free parasites. . . . .	11.9	38.2	31.0	1.31

TABLE IV

*Oxidation of Various Substrates by Preparations from Normal Erythrocytes*

The blood from normal chickens was treated in the same manner as the parasitized blood used to obtain the data of Table II, and the preparations corresponding to parasitized erythrocytes (intact erythrocytes) and to free parasites (solids obtained by hemolysis) were incubated with various substrates under the conditions described in Table II. The values represent the average of two experiments and are expressed in micromoles per hour per cc. of solids obtained by hemolysis.

Substrate added	Oxygen used	
	Intact erythrocytes	Solids obtained by hemolysis
	<i>micromoles</i>	<i>micromoles</i>
None. . . . .	2.4	0.4
Glucose. . . . .	2.2	1.7
Lactate. . . . .	3.0	0.5
Pyruvate. . . . .	2.4	0.4
Succinate. . . . .	3.1	2.6
Malate. . . . .	2.6	0.6

and leucocytes. In general, the activity of the normal preparations is less than 10 per cent of that of the corresponding parasite preparations, and no attempt has been made to correct for the metabolism of the normal blood components.

A number of compounds have been tested for their ability to increase the rate of pyruvate oxidation by free parasites in the presence of all known cofactors, but no active substances have been found. Among the sub-



stances tested and found ineffective were alanine, glutamic acid, horse hemoglobin, acid-denatured horse hemoglobin, proteose peptone, a tryptic digest of casein, adenine, and uracil.

TABLE V  
*Balance of Pyruvate Oxidation*

The samples contained parasitized erythrocytes equivalent to 0.8 cc. of free parasites (carried through the procedure for the preparation of free parasites, except for the addition of antiserum) or 1.4 cc. of free parasites (both preparations from the same sample of blood), 0.01 M pyruvate, 0.00033 M malate, and cofactor mixture, in a volume of 3.0 cc. Gas phase air, temperature 40°, time 60 minutes. The analytical figures are expressed as micromoles per hour per cc. of free parasites. The values designated "corrected for acetate" were calculated by subtracting the oxygen and pyruvate used and the carbon dioxide formed in producing acetate, on the basis of the analytical figures for acetate and the equation  $\text{pyruvate} + \frac{1}{2}\text{O}_2 \rightarrow \text{acetate} + \text{CO}_2$ . The pyruvate completely oxidized was calculated by dividing the oxygen used, corrected for acetate, by 2.5, on the basis of the equation  $\text{pyruvic acid} + 2\frac{1}{2}\text{O}_2 \rightarrow 3\text{CO}_2 + 2\text{H}_2\text{O}$ .

	Parasitized erythrocytes	Free parasites
Oxygen used	52.4	23.2
Total carbon dioxide formed	67.1	31.7
Pyruvate used	25.0	18.6
Acetate formed	0.5	6.5
Pyruvate oxidized to acetate	0.5	6.5
" " completely	20.8	8.0
" accounted for	21.3	14.5
" " " "	85	78
Respiratory quotient		
Observed	1.28	1.37
Corrected for acetate	1.28	1.26
Oxygen-pyruvate ratio		
Observed	2.10	1.25
Corrected for acetate	2.11	1.65
Activity ratio, free-erythrocytic		
Oxygen consumed		0.44
Pyruvate used		0.74

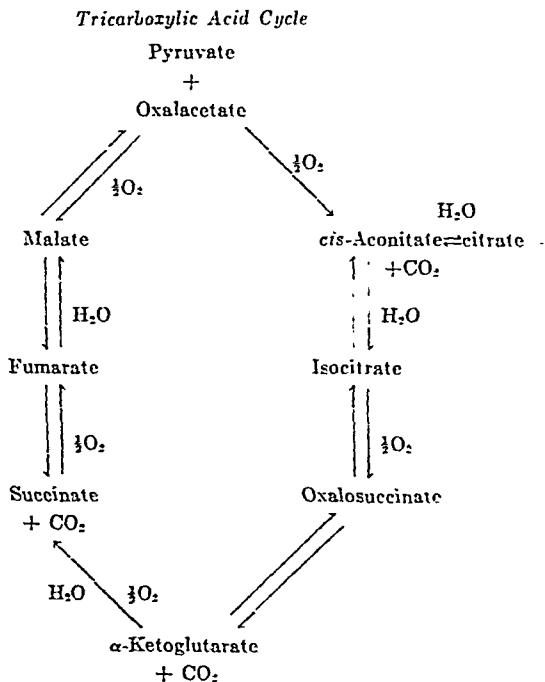
*Balance of Pyruvate Oxidation*

When pyruvate is added to parasite preparations under anaerobic conditions, it does not disappear, but under aerobic conditions pyruvate is rapidly utilized; the oxygen consumption is greatly increased, and the respiratory quotient rises. The major products of pyruvate oxidation are acetate and carbon dioxide and water.

For purposes of comparison, balance experiments were carried out with parasitized erythrocytes and free parasites, both prepared from the same sample of blood and subjected to the same treatment. The results of a

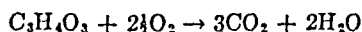
typical experiment of this nature are given in Table V. The parasitized erythrocytes oxidize pyruvate at concentrations of about 0.01 M largely to carbon dioxide and water. Very little acetate is formed, and the values of the respiratory quotient and the oxygen-pyruvate ratio approximate those for complete oxidation (theoretical respiratory quotient 1.2, oxygen-pyruvate ratio 2.5). In contrast, the free parasites form considerable acetate from pyruvate. The respiratory quotient corrected for acetate production is nearly that expected if the pyruvate not converted to acetate is completely oxidized, but the corrected oxygen-pyruvate ratio is low. The percentage of pyruvate accounted for is only slightly less in free parasites than in parasitized erythrocytes. Liberation of the parasites from the erythrocytes results in a greater decrease in the rate of oxygen consumption with pyruvate as substrate than in the rate of pyruvate utilization.

In order to determine the fate of the pyruvate which is not oxidized to acetate or to carbon dioxide and water, analyses have been performed for glucose, acid-hydrolyzable carbohydrate, lactate, succinate, citrate,  $\alpha$ -keto acids, and  $\beta$ -keto acids. However, only very small amounts of citrate and succinate have been found.



*Oxidation of Pyruvate through Tricarboxylic Acid Cycle*

On the basis of extensive investigations, Krebs (4) has proposed a mechanism for the complete oxidation of pyruvate which he has called the tricarboxylic acid cycle. This mechanism is represented in the accompanying diagram. Summation of all the individual reactions of the cycle gives the equation for the complete oxidation of pyruvate.



The experimental work upon which the tricarboxylic acid cycle is based was performed largely with minced pigeon breast muscle. Krebs (4) has listed the fundamental observations which support the tricarboxylic acid cycle mechanism: (1) All the component acids of the tricarboxylic acid cycle are rapidly metabolized by minced pigeon breast muscle. (2) These acids catalyze the oxidation of pyruvate when added to the minced muscle in small amounts. (3) Citrate and  $\alpha$ -ketoglutarate are formed from pyruvate and dicarboxylic acids. (4) Pyruvate oxidation is inhibited by malonate (a specific inhibitor of succinic dehydrogenase) and in the presence of malonate succinate is oxidatively formed from fumarate and pyruvate.

We have performed similar experiments with preparations of *Plasmodium gallinaceum* which indicate that pyruvate is oxidized by means of a tricarboxylic acid cycle in this organism.

*Utilization of Acids of Tricarboxylic Acid Cycle by Parasite Preparations*—Addition of  $\alpha$ -ketoglutarate, succinate, fumarate, malate, or oxalacetate to free parasites causes an increase in oxygen consumption of the same order of magnitude as that produced by the addition of an equivalent quantity of pyruvate (Table VI). *cis*-Aconitate causes a smaller increase in oxygen consumption, and added citrate has very little effect at all, even in the presence of high concentrations of magnesium ions. Krebs (4) has found that oxidation of citrate and *cis*-aconitate in pigeon breast muscle is accelerated by a high concentration of magnesium ion in the medium, but 0.01 M  $\text{MgSO}_4$  only slightly increased the rate of oxidation of these acids in free parasites. The presence of the enzymes fumarase and aconitase has been established by showing the conversion of fumarate to malate and of *cis*-aconitate to citrate in free parasites. The enzyme malic dehydrogenase, requiring diphosphopyridine nucleotide for its action, was demonstrated in extracts of free parasites by the dichlorophenol indophenol technique (3).

In parasitized erythrocytes, only pyruvate, oxalacetate, succinate, and fumarate increase the oxygen consumption (Table VI). The effect of oxalacetate may be due in part to its spontaneous decarboxylation to pyruvate. It is probable that the other acids fail to penetrate the intact erythrocyte in significant amounts.

*Catalysis of Pyruvate Oxidation by Dicarboxylic Acids*—The ability of succinate and other 4-carbon dicarboxylic acids to catalyze the respiration of minced muscle tissue was discovered by Szent-Györgyi and coworkers (36, 37) and confirmed by Stare and Baumann (38). Krebs and Johnson (39) found that citrate acts in a similar catalytic fashion.

We have found that in free parasites oxidative removal of pyruvate is catalyzed by  $\alpha$ -ketoglutarate, succinate, fumarate, malate, and oxalacetate. Typical experiments with malate and  $\alpha$ -ketoglutarate are shown in Table VII. Addition of dicarboxylic acids at a concentration of 0.00033 M to free

TABLE VI

*Effect of Acids of Tricarboxylic Acid Cycle on Respiration of Parasite Preparations*

The samples contained 0.6 cc. of parasitized erythrocytes or 1.2 cc. of free parasites (from different samples of blood) and the substrates indicated below in 0.01 M concentration, in a volume of 3.0 cc. 0.01 M  $\text{MgSO}_4$  was added to all samples containing *cis*-aconitate or citrate. The samples with free parasites all contained the cofactor mixture and also 0.00033 M malate when the substrate was pyruvate. Gas phase air, temperature 40°, time 60 minutes. The values for parasitized erythrocytes are from a single typical experiment, while those for free parasites represent the average of four experiments.

Substrate added	Increase in oxygen consumption over level without added substrates	
	Parasitized erythrocytes	Free parasites
	per cent	per cent
Pyruvate.....	111	167
Oxalacetate.....	51	102
<i>cis</i> -Aconitate.....	0	44
Citrate.....	0	16
$\alpha$ -Ketoglutarate.....	8	88
Succinate.....	18	96
Fumarate.....	16	179
Malate.....	1	204

parasites oxidizing pyruvate characteristically causes an increase in oxygen consumption about twice that required for complete oxidation of the dicarboxylic acid. Total pyruvate utilization is increased and the proportion of pyruvate oxidized to acetate is decreased. In the absence of pyruvate, the increase in oxygen consumption is less than that required for complete oxidation of the dicarboxylic acid.

Catalysis of pyruvate oxidation by acids of the tricarboxylic acid cycle has not been observed in intact parasitized erythrocytes, probably because these acids are already present in optimal amounts.

*Formation of Citrate*—When pyruvate and oxalacetate are incubated aerobically with parasitized erythrocytes or free parasites, a small amount of

citrate is formed (Table VIII). In these experiments, a bicarbonate-saline was used to avoid the clumping of free parasites which occurred when high concentrations of oxalacetate were added to suspensions of free parasites in phosphate-saline. The formation of citrate could not be increased by addition of magnesium or barium ions (40), adenosine triphosphate, malonate, or  $\alpha$ -ketoglutarate (31), by employing a phosphate buffer, or by conducting the experiments under anaerobic conditions. In control experiments it was found that citrate was formed as rapidly by preparations of normal erythrocytes as by preparations of parasitized erythrocytes.

TABLE VII

*Catalysis of Pyruvate Oxidation in Free Parasites by Malate and  $\alpha$ -Ketoglutarate*

The samples contained 1.1 cc. of free parasites, cofactor mixture, and the other additions indicated below, in a total volume of 3.0 cc. Gas phase air, temperature 40°, time 60 minutes. The values are expressed in micromoles.

Substances added	Oxygen used	Pyruvate used	Acetate formed	Pyruvate used corrected for acetate
None	7.3			
1 micromole malate	8.5			
Change caused by malate	+1.2			
30 micromoles pyruvate	14.4	18.5	11.0	7.5
30 " " + 1 micromole malate	19.7	21.6	9.4	12.2
Change caused by malate	+5.3	+3.1	-1.6	+4.7
None	7.3			
1 micromole $\alpha$ -ketoglutarate	9.1			
Change caused by $\alpha$ -ketoglutarate	+1.8			
30 micromoles pyruvate	14.4	18.5	11.0	7.5
30 " " + 1 micromole $\alpha$ -ketoglutarate	19.3	17.6	8.7	8.9
Change caused by $\alpha$ -ketoglutarate	+4.9	-0.9	-2.3	+1.4

However, since the greater part of the solids in parasite preparations consists of the parasites themselves rather than the material present in normal preparations, it is likely that the greater part of the citrate found in parasite preparations was actually formed by the parasites themselves.

*Effect of Malonate on Pyruvate Oxidation*—The inhibition of the enzyme succinic dehydrogenase by malonate was first reported by Quastel and Whetham (41). Subsequent work (36, 42, 43) has confirmed their observation and demonstrated a high degree of specificity for the action of this inhibitor. Szent-Györgyi and coworkers (20, 36) observed that malonate inhibited the respiration of pigeon breast muscle and that succinate was

formed oxidatively from fumarate in the presence of malonate. Further investigations of these effects by Krebs and his associates (22, 39) have furnished some of the strongest evidence for oxidation of pyruvate by the tricarboxylic acid cycle. They found that in the presence of malonate

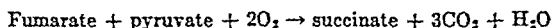
TABLE VIII

*Formation of Citrate in Preparations of Parasitized and Normal Erythrocytes*

The two parasite preparations were made from different samples of blood, the two normal preparations from a single sample. Washings were carried out with bicarbonate-saline (0.025 M  $\text{NaHCO}_3$ , 0.105 M  $\text{NaCl}$ , 0.015 M  $\text{MgSO}_4$ , 0.01 M  $\text{KCl}$ ) gassed with 5 per cent  $\text{CO}_2$ -95 per cent air instead of with phosphate-saline. The samples contained 0.8 cc. of parasitized erythrocytes, 1.2 cc. of free parasites, 1.2 cc. of intact normal erythrocytes, or 1.0 cc. of solids, obtained by hemolysis of normal erythrocytes, 0.025 M  $\text{NaHCO}_3$ , 0.01 M  $\text{MgSO}_4$ , and the substrates indicated below, in a volume of 3.0 cc. Gas phase 5 per cent  $\text{CO}_2$ -95 per cent air, temperature  $40^\circ$ , time 60 minutes. The figures represent micromoles of citrate formed per hour per cc. of intact erythrocytes, free parasites, or solids obtained by hemolysis.

Substrates added	Citrate formed			
	Parasite preparations		Normal erythrocyte preparations	
	Parasitized erythrocytes	Free parasites	Intact erythrocytes	Solids obtained by hemolysis
None	0.0	0.1	0.0	0.0
0.01 M pyruvate + 0.02 M oxalacetate	0.6	0.6	0.8	0.5

succinate was formed from pyruvate and fumarate according to the equation



The only satisfactory explanation which has been proposed for this reaction is that pyruvate condenses with oxalacetate, formed from fumarate to produce a polycarboxylic acid which is oxidized to succinate. Evidence supporting this view has been cited by Krebs (4).

In both parasitized erythrocytes and free parasites, the oxidation of pyruvate is strongly inhibited by 0.02 M malonate (Table IX). Oxygen consumption is inhibited to a greater extent than pyruvate utilization; i.e., the oxidation of pyruvate is less complete. As a result of the addition of malonate, the proportion of pyruvate oxidized to acetate is increased. The greater part of the pyruvate utilized by free parasites in the presence of malonate is oxidized to acetate, and the respiratory quotient and oxygen-pyruvate ratio approach theory for this process (theoretical respiratory quotient 2.0, oxygen-pyruvate ratio 0.5). Thus the mechanism for com-

plete oxidation of pyruvate to carbon dioxide and water may be almost completely inhibited by malonate.

The malonate sensitivity of the succinic dehydrogenase of the malaria parasites was established by the observation that disappearance of succinate added to free parasites is completely inhibited by 0.02 M malonate. In the presence of malonate, succinate is formed by both parasitized eryth-

TABLE IX

*Effect of Malonate on Pyruvate Oxidation in Parasite Preparations*

The samples contained 1.2 cc. of free parasites and cofactor mixture, or 0.5 cc. of parasitized erythrocytes (from a different sample of blood), 0.02 M pyruvate, and 0.02 M malonate, as indicated below, in a volume of 3.0 cc. Gas phase air, temperature 40°, time 90 minutes for parasitized erythrocytes and 60 minutes for free parasites. The values are expressed as micromoles per hour per cc. of parasitized erythrocytes or free parasites. The corrections for acetate are described in Table V

	Parasitized erythrocytes		Free parasites	
	Pyruvate	Pyruvate + malonate	Pyruvate	Pyruvate + malonate
Oxygen used	37.2	18.6	19.8	8.2
Carbon dioxide formed . . .	47.5	28.4	29.5	14.1
Pyruvate used	21.6	19.0	18.7	12.5
Acetate formed	0.0	4.1	8.0	10.1
Succinate formed	0.0	0.0	1.0	1.2
Respiratory quotient .	1.27	1.53	1.49	1.72
Oxygen-pyruvate ratio	1.73	0.98	1.06	0.66

% inhibition				
Oxygen used, total . . .		50		59
“ “ corrected for acetate		56		84
Pyruvate used, total		12		33
“ “ corrected for acetate		31		76

rocyte and free parasites (Table X). A small amount of succinate accumulates in the absence of added substrates. Addition of pyruvate increases succinate accumulation; the amount of succinate formed from pyruvate alone is presumably limited by the quantity of dicarboxylic acids present in the parasite preparations and available for condensation with pyruvate. Fumarate or malate alone also gives rise to succinate in the presence of malonate; they may be oxidized to oxalacetate, which is partially decarboxylated to pyruvate, thus forming the other component of the condensation reaction. When pyruvate and a dicarboxylic acid are added together, inhibition of oxygen consumption by malonate is

reduced or completely abolished, and maximum yields of succinate are obtained. Under these conditions, the succinate formed in free parasites represents 50 to 60 per cent of the pyruvate and malate utilized. The fact that succinate accumulates in free parasites in the absence of malonate

TABLE X

*Oxidative Formation of Succinate by Parasite Preparations*

The samples contained 1.0 cc. of parasitized erythrocytes or 1.2 cc. of free parasites and the other additions indicated below, in a volume of 4.0 cc. The substrates were added at a concentration of 0.01 M and malonate at a concentration of 0.02 M. Fumarate was used as a substrate with parasitized erythrocytes instead of malate because it penetrates the erythrocyte more readily. Gas phase air, temperature 40°, time 120 minutes for parasitized erythrocytes and 60 minutes for free parasites. The values are expressed as micromoles per hour per cc. of parasitized erythrocytes or free parasites. The values for succinate actually represent the sum of succinate and  $\alpha$ -ketoglutarate, since the determinations were carried out after heating the samples with acid permanganate to destroy malonate, a procedure which oxidizes  $\alpha$ -ketoglutarate to succinate (22).

Substrates added	Malonate added	Oxygen used	Pyruvate used	Malate used	Succinate formed
Parasitized erythrocytes					
None.....	—	14.0			0.0
	+	6.5			3.7
Pyruvate.....	—	42.3	17.7		1.1
	+	14.5	17.7		4.6
Fumarate.....	—	18.1			0.0
	+	11.3			3.0
Pyruvate + fumarate.....	—	42.7	14.7		1.0
	+	23.4	18.0		3.9
Free parasites					
None.....	—	5.1			0.0
	+	1.4			0.2
Pyruvate.....	—	17.1	19.7		0.0
	+	10.5	19.7		1.5
Malate.....	—	17.1		17.6	2.2
	+	15.7		16.4	4.9
Pyruvate + malate.....	—	28.5	18.8	17.6	10.5
	+	26.3	19.0	17.6	11.5

inhibition of the succinic dehydrogenase suggests that the oxidation of succinate may be a limiting step in the oxidation of carbohydrate by free parasites.

It was found that malonate inhibits the oxidation of glucose and lactate to approximately the same extent as that of pyruvate (Table XI). This



observation indicates that these substances are oxidized by the same mechanisms as is pyruvate itself.

### *Oxidation of Pyruvate to Acetate*

Parasitized erythrocytes and free parasites oxidize pyruvate to a volatile acid which was identified as acetic acid. Duclaux distillation of concen-

TABLE XI

#### *Effect of Malonate on Oxidation of Different Substrates*

The conditions were similar to those described in Table IX, except that the experimental period was only 30 minutes.

Substrate added	Per cent inhibition of oxygen consumption by 0.02 M malonate	
	Parasitized erythrocytes	Free parasites
0.015 M glucose . . . . .	69	35
0.030 " <i>dl</i> -lactate . . . . .	67	67
0.015 " pyruvate . . . . .	61	51

TABLE XII

#### *Effect of Pyruvate Concentration on Acetate Formation*

The samples contained 0.5 cc. of parasitized erythrocytes or 1.0 cc. of free parasites (from different samples of blood) and the other additions indicated below, in a volume of 3.0 cc. The cofactor mixture and 0.00033 M malate were added to samples containing free parasites. Gas phase air, temperature 40°, time 60 minutes. The values are expressed as micromoles per hour per cc. of parasitized erythrocytes or free parasites.

	Parasitized erythrocytes		Free parasites	
	0.01 M pyruvate	0.03 M pyruvate	0.01 M pyruvate	0.03 M pyruvate
Oxygen used . . . . .	61.5	61.9	21.6	17.4
Carbon dioxide formed . .	76.0	80.9	31.7	27.1
Pyruvate used . . . . .	32.4	41.5	20.5	19.6
Acetate formed . . . . .	0.9	5.6	8.4	10.6
Respiratory quotient . . .	1.23	1.31	1.47	1.56
Oxygen-pyruvate ratio . . .	1.90	1.49	1.05	0.89

trates of the volatile acid indicated that the substance was acetic acid slightly contaminated with lactic acid from the distillation reagent. The concentrates gave a strong positive lanthanum nitrate test for acetate (44) and gave a positive test for acetaldehyde when heated with calcium formate, according to the directions of Feigl (45).

Free parasites form large amounts of acetate even at low pyruvate concentrations, but appreciable quantities of acetate are produced by para-

sitized erythrocytes only when the pyruvate concentration is high (Table XII). In the presence of malonate, both types of preparations produce acetate at low pyruvate levels (Table IX). Acetate is formed from pyruvate only under aerobic conditions (Table XIII). Dismutation of pyruvate to produce lactate, acetate, and carbon dioxide does not occur. The formation of acetate from glucose and lactate in free parasites (Table XIII) offers further evidence for the belief that these substances are oxidized through pyruvate as an intermediate.

Addition of acetate to parasite preparations causes no change in the rate of oxygen consumption, and the acetate does not disappear (Table XIII). It thus appears that acetate is an end-product in the carbohydrate metabolism of the malaria parasite.

TABLE XIII

*Formation and Disappearance of Acetate in Parasite Preparations*

The experimental conditions were like those described in Table XII. The values are expressed in micromoles per hour per cc. of parasitized erythrocytes or free parasites.

Substrate added	Gas phase	Change in acetate	
		Parasitized erythrocytes	Free parasites
None.....	Air	+0.9	0.0
0.01 M acetate.....	"	0.0	0.0
0.01 " pyruvate.....	Nitrogen	0.0	0.0
0.01 " ".....	Air	+0.9	+10.2
0.01 " glucose.....	"	+0.3	+4.5
0.02 " dl-lactate.....	"	+0.2	+3.6

## DISCUSSION

The observations concerning the cofactors for pyruvate oxidation in the free parasites are of considerable interest. The finding that manganous ions stimulate pyruvate oxidation is in agreement with previous work on the participation of manganese in enzyme systems metabolizing pyruvate (46),  $\alpha$ -ketoglutarate (47), oxalacetate (48), and oxalosuccinate (49, 50). The acceleration obtained with adenosine triphosphate suggests that pyruvate oxidation in the parasites is linked with the esterification of inorganic phosphate, although this has not been demonstrated directly. The stimulatory effects of thiamine and diphosphothiamine provide evidence for the participation of thiamine in the metabolism of malaria *Plasmodia*. From investigations carried out with enzymes from other sources, it is known that diphosphothiamine acts as a coenzyme for the oxidation and decarboxylation of pyruvate and other keto acids (51), although the mech-

anism of this action is not clear. It is probable that the two pyridine nucleotides, containing nicotinamide, accelerate pyruvate oxidation by serving as coenzymes for the malic and isocitric dehydrogenases which participate in the tricarboxylic acid cycle. By enzymic assay (50), we have found that the concentration of diphosphopyridine nucleotide in free parasites is about 0.00002 M.

Previous work has demonstrated that other water-soluble vitamins and derivatives are involved in the metabolism of malaria parasites. Trager (52) has observed that calcium pantothenate favors the survival of *Plasmodium lophurae in vitro*, and it has been found that analogues of pantothenic acid, such as pantooyltaurine, exhibit antimalarial activity against *P. gallinaceum*.<sup>5</sup> Trager (52) also obtained positive results with biotin in his parasite survival experiments. Ball and his coworkers (53) have found that *p*-aminobenzoic acid has a strong stimulatory effect on the *in vitro* growth of *P. knowlesi*, and they have demonstrated the presence in this organism of considerable quantities of riboflavin as flavin-adenine-dinucleotide. Therefore, at least six water-soluble vitamins, thiamine, nicotinamide, riboflavin, pantothenic acid, biotin, and *p*-aminobenzoic acid, serve some metabolic function in malaria parasites.

The evidence presented in this paper indicates clearly that the oxidation of pyruvate by *Plasmodium gallinaceum* involves the tricarboxylic acid cycle. All the intermediate acids of the cycle are readily utilized by the parasite, with the exception of citrate and *cis*-aconitate. No explanation can be offered for the relative inertness of these substances; in the case of *cis*-aconitate, at least, it is not due to failure to penetrate the parasite cell, since added *cis*-aconitate was converted to citrate by free parasites. Catalysis of pyruvate oxidation can be demonstrated with all the acids of the tricarboxylic acid cycle, again with the exception of citrate and *cis*-aconitate. The evidence for the formation of citrate from pyruvate and dicarboxylic acids is unsatisfactory because of the relatively large formation of citrate by normal erythrocyte preparations. At most only very small amounts of citrate seem to be produced by the parasites. In pigeon breast muscle Krebs and Eggleston (22) could obtain citrate formation equivalent to only about 15 per cent of the fumarate added in the presence of excess pyruvate. It is now believed that citrate is not a direct intermediate in the tricarboxylic acid cycle, but that it may accumulate when utilization of the other tricarboxylic acids is slower than their formation. The marked malonate inhibition of pyruvate oxidation and the oxidative formation of succinate from pyruvate and dicarboxylic acids in the presence of malonate provide the strongest evidence for the tricarboxylic acid cycle in the malaria parasites.

<sup>5</sup> Personal communication from Mr. William Cantrell.

The question of the importance of oxidation of pyruvate to acetate is of some interest. Acetate was consistently formed from pyruvate by free parasites, and acetate formation could be demonstrated in parasitized erythrocytes and increased in free parasites by the use of high pyruvate concentrations or by the addition of malonate. Catalytic amounts of dicarboxylic acids decreased the formation of acetate in free parasites. Two possible explanations of these facts may be proposed. The first explanation is based on the view that the initial step in the oxidation of pyruvate is the formation of a 2-carbon particle (54, 55). Once formed, the 2-carbon unit may condense with oxalacetate and be oxidized through the tricarboxylic acid cycle, or it may be converted to acetate by unknown mechanisms. If the rate of formation of the 2-carbon unit is increased by high pyruvate concentrations beyond the capacity of the tricarboxylic acid cycle to oxidize it, acetate is formed. Or if the tricarboxylic acid cycle mechanism is blocked by malonate, the 2-carbon particle is converted to acetate. It is very likely that damage to the enzymic mechanism of the tricarboxylic acid cycle in free parasites is responsible for the formation of acetate from pyruvate by these preparations. Addition of small amounts of dicarboxylic acids, by partially restoring the tricarboxylic acid cycle mechanism, decreases the proportion of the 2-carbon particle converted to acetate. This explanation visualizes essentially a competition between two systems for the 2-carbon unit formed from pyruvate, with oxidation through the tricarboxylic acid cycle predominating. As an alternative explanation, two completely independent pathways of pyruvate metabolism may be assumed to exist—one the tricarboxylic acid cycle, and the other the formation of acetate, perhaps by a reaction like that described in bacteria by Lipmann (7, 56). These two mechanisms might then compete for the intact pyruvate molecule in the manner indicated previously for the 2-carbon particle derived from pyruvate. The available data do not make it possible to distinguish between the two schemes in the malaria parasite. However, it is unlikely that acetate formation is an important metabolic pathway under physiological conditions, *i.e.* in malaria parasites utilizing glucose while within the intact erythrocyte.

The metabolic similarities and differences displayed by parasitized erythrocytes and free parasites deserve some mention. The glycolytic activity of the parasites is the same whether they are in the intact erythrocyte or are free. Malonate inhibition of pyruvate oxidation and formation of acetate from pyruvate under suitable conditions can be demonstrated with both types of preparations. However, in contrast to the free parasites, intact parasitized erythrocytes do not readily oxidize certain acids of the tricarboxylic acid cycle (malate,  $\alpha$ -ketoglutarate, and *cis*-aconitate), do not show an acceleration of pyruvate oxidation by dicarboxylic acids and other cofactors, and do not form acetate at low concentrations of pyruvate.

These differences can be explained by three factors. First, substances such as phosphorylated compounds, tricarboxylic acids, and dicarboxylic acids with extra polar groups (malate or  $\alpha$ -ketoglutarate) may not be able to penetrate the erythrocyte because of the relative impermeability of the erythrocyte to highly polar substances (57). Second, intact parasitized erythrocytes may contain adequate quantities of dicarboxylic acids and other cofactors, but these substances may be lost when the erythrocytes are destroyed and the parasites washed extensively. Third, the enzymic mechanism of the tricarboxylic acid cycle may be damaged in the process of isolating the free parasites; in particular, the mechanism for the oxidation of succinic acid seems relatively inactive.

A common pathway for the oxidation of glucose, lactate, and pyruvate by the malaria parasite is indicated by the malonate inhibition and acetate formation observed in the oxidation of all three substances. Accordingly, it is now possible to indicate the following pathway for carbohydrate utilization by these organisms: glucose is converted to pyruvate by a typical phosphorylating glycolytic mechanism; the pyruvate is oxidized to carbon dioxide and water through the tricarboxylic acid cycle or is reversibly reduced to lactate. Even under aerobic conditions, lactate accumulates during the glycolysis of glucose. This fact suggests that dihydrodiphosphopyridine nucleotide formed during glycolysis is reoxidized more rapidly by pyruvate than by the hydrogen transport systems leading to molecular oxygen. The lactate formed in this manner is subsequently reoxidized to pyruvate. There may be other pathways for utilization of pyruvate by the parasites, but, with the exception of the conversion of pyruvate to acetate under certain conditions, these have not yet been demonstrated.

#### SUMMARY

1. A method employing specific hemolytic serum for the preparation of free malaria parasites from chicken blood infected with *Plasmodium gallinaceum* is described.
2. Free parasites oxidize pyruvate at a maximum rate only in the presence of dicarboxylic acids such as succinate, thiamine or diphosphothiamine, diphosphopyridine nucleotide, triphosphopyridine nucleotide, adenosine triphosphate, and manganous ions. Intact parasitized erythrocytes do not require cofactors.
3. The ability of free parasites to oxidize various substrates is about half that of parasitized erythrocytes.
4. Pyruvate is oxidized almost completely to carbon dioxide and water by parasitized erythrocytes, but with free parasites appreciable quantities of acetate are also formed.

5. The parasites utilize succinate, fumarate, malate, oxalacetate, *cis*-aconitate, and  $\alpha$ -ketoglutarate; the oxidation of pyruvate is catalyzed by the dicarboxylic acids and is strongly inhibited by malonate, with the accumulation of succinate. It is concluded that the mechanism for complete oxidation of pyruvate involves the tricarboxylic acid cycle.

6. Parasitized erythrocytes oxidize pyruvate to acetate only at high concentrations of pyruvate, while free parasites form acetate at lower pyruvate concentrations. Malonate inhibition increases acetate formation in both preparations. Added acetate is not utilized.

7. Oxidation of glucose and lactate by the parasites gives rise to acetate and is inhibited by malonate. It is concluded that these substances are oxidized by the same path as pyruvate.

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# THE BIOCHEMISTRY OF THE MALARIA PARASITE

## VI. STUDIES ON THE NITROGEN METABOLISM OF THE MALARIA PARASITE\*

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Little is known concerning the nitrogen metabolism of the malaria parasite or of its nitrogen requirements. Christophers and Fulton (1) have made limited observations regarding these questions, but no systematic studies have been published.

Since the erythrocytic forms of the malaria parasite rapidly increase in size and number in the blood of infected animals, the requirement of nitrogen for the synthesis of parasite protein is undoubtedly great. The hemoglobin of parasitized erythrocytes is a readily available source of nitrogen for the parasites, and the presence of large amounts of hematin in infected erythrocytes (2) suggests that the malaria parasite has the ability to break down hemoglobin during the course of its metabolism.

The results of the present investigation support such a view. It was found that large amounts of amino nitrogen are liberated during the *in vitro* incubation of chicken erythrocytes infected with *Plasmodium gallinaceum*. Furthermore, cell-free extracts of the malaria parasites themselves have been prepared which hydrolyze added hemoglobin or globin.

### Methods

*Parasite Preparations*—Parasitized erythrocytes were prepared as described in Paper V (3). Unless otherwise specified, the phosphate-saline used in washing the erythrocytes contained 0.1 per cent glucose.

*Cell-free extracts* of *Plasmodium gallinaceum* were prepared by methods resembling those already described (4). All operations were carried out in a cold room at 0°. Parasitized erythrocytes, prepared as indicated above, were mixed with 2 volumes of water and shaken several times during a period of 15 minutes. At this time, hemolysis was complete, and tonicity was restored by the addition of 0.1 volume of 7 per cent sodium chloride. The parasites, which had undergone little cytolysis during treatment with

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water, were centrifuged down at 2000*g* and washed four times with 5 to 8 volumes of Ca-free phosphate-saline containing 0.1 per cent glucose by centrifugation at 2000*g* for 10 minutes. This washing removed almost all of the hemoglobin from the preparation. Powdered Pyrex glass (100 mesh) was then added to the parasites until a thin paste was formed, and the mixture was ground in a small bacterial mill (5). After grinding, the parasite material was shaken continuously for 30 minutes with 1 volume of water. The mixture was centrifuged 15 minutes at 2000*g*, and a supernatant liquid was obtained which contained the parasite proteinases in solution. The appearance of the extract varied from dark and cloudy to faint pink and almost clear without relation to the proteinase activity. Microscopic examination showed the absence of intact parasites. The extract showed only faint traces of hemoglobin, when examined with a hand spectroscope. 2 cc. of cell-free extract represent the material extracted from approximately 1 cc. of parasitized erythrocytes.

*Analytical Methods*—Amino nitrogen was determined on tungstate filtrates by the colorimetric method of Frame, Russell, and Wilhelmi (6, 7). In some experiments, amino nitrogen was determined both by the colorimetric method and by the specific ninhydrin method of Hamilton and Van Slyke (8). The values obtained with these two methods checked within reasonable limits. Since parasitized erythrocytes often produced appreciable quantities of ammonia, the method of Frame *et al.* was modified for use in the presence of this interfering substance. It was necessary to remove the ammonia because it gave highly variable results in the colorimetric amino nitrogen method, and suitable corrections could not be applied. After it was found that short periods of aeration failed to remove all of the ammonia from the amino nitrogen samples, a method of vacuum distillation was adopted which was similar to the procedure used by Archibald (9) for the microdetermination of ammonia. The following modified amino nitrogen method was used in all of the experiments described in this paper. The determinations were carried out in 25 × 200 mm. Pyrex test-tubes graduated at 5 and at 15 cc. Appropriate aliquots of the tungstate filtrates were added to the graduated tubes and neutralized to phenolphthalein, as in the original procedure. After the neutralized aliquots were diluted to 5 cc., 3 drops of 0.1 *N* NaOH were added to each tube to bring the pH to about 10.0 to 10.5. A 2-hole rubber stopper carrying a fine capillary extending almost to the bottom of the tube and an outlet tube of 10 mm. diameter was placed in each tube. The tubes were then placed in a water bath at 60° in groups of four. By means of suitable manifolds, the capillaries were connected to a sulfuric acid trap open to the air, and the outlet tubes were connected to an efficient water aspirator. The capillary openings were of such a diameter that when four tubes were

connected to one aspirator, a pressure of about 50 mm. of mercury was produced. At 50 mm. pressure and 60° external temperature, the contents of the tubes boiled rapidly, bumping being prevented by the fine stream of air entering from the capillaries. Depending upon the exact dimensions of the apparatus, 2 to 4 cc. of water distilled from each tube in 5 to 10 minutes, carrying all of the ammonia with it. A trial run served to indicate the exact distillation time necessary for complete removal of ammonia. At the end of the distillation, the vacuum was released and the tubes were removed from the bath. The capillary was washed with 1 cc. of water, and the tube walls were rinsed down with sufficient water to bring the volume back to 5 cc. The solution was made acid to phenolphthalein with a drop of 0.5 N HCl, and 0.1 N NaOH was again added, until a permanent phenolphthalein pink was produced. Color was then developed, according to the original directions (6) as modified by Russell (7). The vacuum distillation removed all the ammonia and did not alter the amino nitrogen content of the samples. The volume of the sample during distillation must not be allowed to fall below 1 cc. or the phenolphthalein will form compounds in the alkaline solution which give color in the final sample.

*Ammonia* was determined by distillation of tungstate filtrates in a Pregl apparatus, followed by nesslerization of the distillates. By this method, 5 to 20  $\gamma$  of ammonia could be estimated with a precision of 3 to 5 per cent. The tungstate filtrate was measured into a standard Pregl distillation apparatus, made alkaline with 5 cc. of 10 per cent NaOH, and distilled into a 16  $\times$  150 mm. Pyrex test-tube graduated at 10 cc. and calibrated as a colorimeter tube. This receiver contained 2 cc. of 1 per cent boric acid. When 6 cc. of distillate were collected, distillation was stopped. 1 cc. of modified Nessler's reagent (10) was added, and the contents of the tube were mixed and diluted to 10 cc. Color was read in an Evelyn photoelectric colorimeter with a No. 420 filter against a blank and standards also containing 2 cc. of 1 per cent boric acid. A special sleeve was used to accommodate the small colorimeter tubes.

*Total non-protein nitrogen* was determined on tungstate filtrates by use of the micro-Kjeldahl digestion procedure of Koch and McMeekin (11). The sulfuric acid used in digestion was neutralized with an equivalent amount of NaOH and the samples were nesslerized and read as in the determination of ammonia.

*Manometric Methods*—Oxygen consumption was measured in a Barcroft-Warburg apparatus. The center cup of each Warburg vessel contained 0.2 cc. of 20 per cent KOH placed on a roll of filter paper. When anaerobic conditions were desired, a stick of yellow phosphorus was placed in the center cup of the vessel, which was flushed out with tank nitrogen.

*Preparation of Substrates*—Horse oxyhemoglobin was crystallized by the method of Heidelberger (12), recrystallized one time, and dried under reduced pressure at 0°. Chicken hemoglobin solutions were prepared from well washed chicken erythrocytes by hemolyzing the erythrocytes with water, removing the solids by centrifugation, and dialyzing against distilled water. To obtain denatured globin, the hemoglobin preparations were treated with 0.1 N HCl for 5 minutes at room temperature, and the acid-denatured globin was precipitated with acetone. After exhaustive washing with acetone, the globin was dried at reduced pressure. When the acetone-dried denatured globin was resuspended in water and neutralized to pH 6.5, about half of the protein remained in solution. The concentration of all protein solutions was determined by micro-Kjeldahl analysis.

## Results

### *Nitrogen Metabolism of Parasitized Erythrocytes*

*Formation of Amino Nitrogen and Ammonia*—Chicken erythrocytes infected with *Plasmodium gallinaceum*, consistently liberated large amounts of amino nitrogen while metabolizing glucose in air, while normal erythrocytes showed no change in amino nitrogen under identical conditions. Neither normal nor parasitized erythrocytes formed appreciable quantities of ammonia (Table I). Because of the small quantities involved, positive identification of the substances formed during the metabolism of parasitized erythrocytes which were determined as amino nitrogen could not be made. However, the data of Table II show that most of the material was  $\alpha$ -amino nitrogen (ninhydrin carboxyl nitrogen) and that the changes in amino nitrogen accounted for the large fraction of the change in total non-protein nitrogen. The rate of amino nitrogen formation generally decreased throughout the incubation period, although the rate of oxygen consumption usually remained constant.

The average initial rate of amino nitrogen liberation by parasitized erythrocytes was equivalent to about 1 mg. of amino acids per hour per cc. of erythrocytes. The only proteins of the chicken erythrocyte which are present in amounts sufficient to serve as substrates for such a rate of hydrolysis are hemoglobin and nucleoprotein. Although it has not been definitely proved, it is highly probable that hemoglobin is the substrate from which amino nitrogen is formed by the malaria parasite because large deposits of hemozoin are present in erythrocytic parasites (2) and because microscopic examination of the nuclei of parasitized chicken erythrocytes does not show evidence of damage or disintegration.

The production of amino nitrogen and ammonia by parasitized erythrocytes is greatly influenced by changes in their environment. The

presence or absence of glucose, oxygen, and the antimalarial drugs, quinine and atabrine, caused characteristic changes in the nitrogen metabolism of the malaria parasite.

*Effect of Glucose*—In air and in the absence of glucose, parasitized erythrocytes consumed oxygen at a slowly declining rate for as long as 4

TABLE I

*Formation of Amino Nitrogen and Ammonia by Normal and Parasitized Chicken Erythrocytes*

Each Warburg vessel contained 0.4 to 0.7 cc. of chicken erythrocytes and 0.01 M glucose in 3.0 cc. of Ca-free phosphate-saline. The center cup of each vessel contained 0.2 cc. of 20 per cent KOH. All the vessels were shaken in air at 40° for 4 hours. The values are expressed as micromoles per cc. of erythrocytes. The expression of variation is the average deviation from the arithmetical mean.

	Normal erythrocytes (3 experiments)	Parasitized erythrocytes (6 experiments)
Amino nitrogen, initial.....	21.3	24.1
“ “ final.....	22.2	50.4
“ “ increase.....	0.9 ± 0.7	26.3 ± 6.5
Ammonia nitrogen, initial.....	2.1	2.6
“ “ final.....	3.1	3.4
“ “ increase.....	1.0 ± 0.8	0.8 ± 1.1
Oxygen consumption.....	9 ± 2	125 ± 25

TABLE II

*Changes in Non-Protein Nitrogen Fractions of Parasitized Erythrocyte Suspensions*

The experimental conditions were identical with those described in Table I. Air, 40°, 4 hours, 0.01 M glucose. The values are expressed as micromoles per cc. of erythrocytes.

	Initial	Final	Increase
Total non-protein nitrogen.....	69.6	122.0	52.4
Colorimetric amino “.....	32.8	61.6	28.8
Ninhydrin α-amino “.....	21.9	45.4	23.5
Ammonia nitrogen.....	1.3	4.4	3.1

hours. At the same time, large amounts of ammonia appeared. In the absence of glucose, the increase in ammonia nitrogen was paralleled by an equivalent decrease in the amino nitrogen accumulation as compared with a control sample containing glucose; *i.e.*, the sum of the increase in amino nitrogen plus ammonia nitrogen was the same in the presence and in the absence of glucose (Table III). This equivalence was not fortuitous, but was observed each time such an experiment was performed. On the basis

of these results, it may reasonably be assumed that the malaria parasite can deaminate amino acids.

It is unlikely that the ammonia formed in the absence of glucose was produced after the death of the parasite. First, as already mentioned, the oxygen consumption of glucose-free suspensions was considerable and declined but slowly during the period of observation. Second, after 4 hours aerobic incubation in the absence of glucose, parasitized erythrocytes were almost as infective when injected into 2 week-old chicks as were equivalent samples incubated aerobically in the presence of glucose for the same time.

TABLE III

*Effect of Glucose on Nitrogen Metabolism of Parasitized Erythrocyte Suspensions*

The parasitized erythrocytes were washed and suspended in Ca-free phosphate-saline to which no glucose had been added. In the samples which were incubated without glucose, the glucose solution was replaced by an equal volume of Ca-free phosphate-saline. Air, 40°, 4 hours. The values are given as micromoles per cc. of erythrocytes.

0.01 M glucose added	Amino nitrogen formed	Ammonia nitrogen formed	Amino nitrogen plus ammonia nitrogen formed	Oxygen used
Experiment 1				
+	33.3	7.7	41.0	153
-	23.0	20.0	43.0	76
Experiment 2				
+	23.3	1.4	24.7	109
-	15.8	8.4	24.2	53

*Effect of Anaerobic Conditions*—Further investigation of the formation of amino nitrogen revealed that the process is not one of simple protein hydrolysis. Under anaerobic conditions, liberation of amino nitrogen by parasitized erythrocytes was inhibited 40 to 80 per cent, both in the presence and absence of glucose (Table IV). This effect, which is not observed during ordinary enzymic hydrolysis of proteins, indicates that the liberation of amino nitrogen by the malaria parasite is related in some manner to oxidative processes.

The effect of anaerobiosis upon ammonia production was less pronounced, but lack of oxygen definitely prevented the sharp rise in ammonia formation caused by the absence of glucose (Table IV).

*Effect of Antimalarial Drugs*—When added to parasitized erythrocytes, quinine and atabrine inhibited the production of amino nitrogen in concentrations as low as  $3.3 \times 10^{-5}$  M (Table V). Atabrine consistently inhibited amino nitrogen liberation at all higher concentrations, and the

inhibition was proportional to the concentration of atabrine present. However, the effect of quinine was more complex. At most concentrations, quinine inhibited amino nitrogen accumulation to about the same extent as did atabrine; but in concentrations of about  $1 \times 10^{-4}$  M, quinine characteristically caused an increased accumulation of amino nitrogen. One possible

TABLE IV

*Effect of Anaerobic Conditions upon Formation of Amino Nitrogen and Ammonia in Parasitized Erythrocytes*

The data represent the same experiment as Experiment 2 of Table III. The anaerobic conditions were obtained by introducing nitrogen into the vessels and by placing yellow phosphorus sticks in the center cups. 40°, 4 hours. The values are given as micromoles per cc. of parasitized erythrocytes.

0.01 M glucose added	Fraction	Amount formed in		Inhibition in nitrogen
		Air	Nitrogen	
+	Amino nitrogen	23.3	4.2	82
+	Ammonia nitrogen	1.4	3.3	-120
-	Amino nitrogen	15.8	3.7	80
-	Ammonia nitrogen	8.4	3.0	64

TABLE V

*Effect of Quinine and Atabrine on Formation of Amino Nitrogen in Parasitized Erythrocytes*

The experiment was carried out under the conditions described in Table I. Quinine and atabrine were used as the dihydrochlorides to which 1.5 equivalents of NaHCO<sub>3</sub> solution saturated with CO<sub>2</sub> had just been added. Air, 40°, 4 hours, 0.01 M glucose. The control production of amino nitrogen was 21.7 micromoles per cc. of erythrocytes.

Concentration of inhibitor	Inhibition produced	
	Quinine	Atabrine
M	per cent	per cent
$1.0 \times 10^{-3}$	67	65
$3.3 \times 10^{-4}$	54	46
$1.0 \times 10^{-4}$	-19	31
$3.3 \times 10^{-5}$	18	21

explanation of this unexpected observation is discussed in the final section of the paper.

Quinine and atabrine effectively inhibited amino nitrogen formation in concentrations lower than those necessary for any *in vitro* effect other than the inhibition of the oxygen consumption of parasitized erythrocytes (13). The lowest concentrations of quinine and atabrine ( $3 \times 10^{-5}$  M) which

inhibited amino nitrogen production were only slightly higher than the concentrations of these antimalarials in the erythrocytes of chickens receiving therapeutically effective dosages (14, 15).

*Coupling of Protein Hydrolysis with Oxidative Processes of Malaria Parasite*—The sensitivity of amino nitrogen production to anaerobic conditions and to antimalarial drugs suggests that these effects are brought about only indirectly, and that the primary effects are upon oxidative processes, which, in some manner, are necessary for a maximum rate of protein hydrolysis in the malaria parasite.

### *Proteinase Activity of Cell-Free Extracts of Malaria Parasite*

The hypothesis just suggested was tested by studying protein hydrolysis in cell-free extracts of the malaria parasite. In such a simplified, non-respiring system any effect of anaerobiosis or of quinine and atabrine upon protein hydrolysis would indicate a direct effect upon the parasite proteinases themselves. Conversely, the failure of these factors to influence the rate of protein hydrolysis in cell-free extracts would constitute evidence for the existence of an oxidative coupling mechanism in the intact malaria parasite.

*Characteristics of Proteinase System in Cell-Free Extracts*—Cell-free extracts of *Plasmodium gallinaceum*, prepared as described earlier in the paper, contained an active proteinase or system of proteinases. Proteinase activity was measured in terms of amino nitrogen liberation. In the presence of excess substrate, the rate of protein hydrolysis was linear for at least 3 hours (Fig. 1, A) and was proportional to the amount of cell-free extract present (Fig. 1, B). The activity of the extracts was somewhat higher at pH 6.5 than at pH 7.4, and the experiments were carried out at the more acid pH.

In the absence of added substrate, little protein hydrolysis occurred. Added horse hemoglobin was split very slowly or not at all, but acid-denatured horse globin was rapidly hydrolyzed (Table VI). A more rapid rate of hydrolysis of denatured protein than of native protein is frequently observed in enzymic hydrolysis (16), and this behavior should not be interpreted as a peculiar property of the parasite proteinases. Chicken hemoglobin and globin, the proteins available to *Plasmodium gallinaceum* in its avian host, were hydrolyzed at the same rate as were horse hemoglobin and globin.

The addition of cofactors such as cyanide or manganous salts, which are often necessary for the activity of intracellular proteinases, caused no increase in the proteinase activity of the parasite extracts. In fact, both these substances were strongly inhibitory.

The cell-free extracts consumed no oxygen and produced no ammonia, either in the presence or absence of globin or of *dl*-alanine.

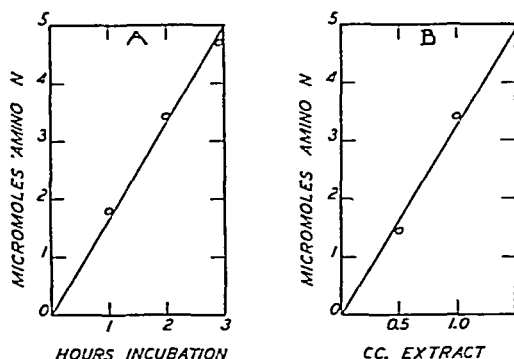


Fig. 1. Hydrolysis of denatured horse globin by cell-free extracts of the malaria parasite; (A) the effect of time of incubation upon rate of hydrolysis; (B) the effect of concentration of cell-free extract upon rate of hydrolysis. Small test-tubes were filled with 1.0 cc. of cell-free extract (or extract plus sufficient water to make 1.0 cc.), 0.25 cc. of 0.1 M phosphate buffer, pH 6.5, and 0.25 cc. of denatured horse globin solution, pH 6.5, containing 200 micromoles of total nitrogen. A drop of toluene was added to each tube and all were incubated at 40° for the times indicated in A and for 2 hours to obtain the values given in B. Protein hydrolysis was measured in terms of increase in amino nitrogen.

TABLE VI

*Proteinase Activity of Cell-Free Extracts of Malaria Parasite*

1.0 cc. of cell-free extract and 0.25 cc. of 0.1 M phosphate, pH 6.5, were incubated 2 hours at 40°. Hemoglobin and globin were added in amounts of 200 micromoles of total nitrogen. The values are expressed as micromoles of amino nitrogen per cc. of extract and represent the average of five experiments.

Amino nitrogen	Substrates added		
	None (1)	Native horse hemo- globin (2)	Denatured horse globin (3)
Initial.....	0.66	0.66	0.85
Final.....	1.02	1.41	7.16
Increase.....	0.37	0.75	6.31
S.D.....	0.30	0.47	1.51
P*.....		0.05-0.10	< 0.01

\* P is a measure of the probability that the difference between two sets of measurements (here (1) and (2) and (1) and (3)) is due only to chance variation; i.e., a P of 0.01 indicates that only one time in 100 will the difference between two sets of measurements be due solely to chance.

*Effect of Anaerobiosis and of Antimalarials*—In sharp contrast to amino nitrogen formation in intact parasitized erythrocytes, the liberation of



amino nitrogen by cell-free extracts was not decreased under anaerobic conditions (cf. Tables IV and VII).

In concentrations of  $1 \times 10^{-3}$  M, quinine and atabrine significantly inhibited the activity of the parasite proteinase (Table VII). Lower concentrations of antimalarials were without effect. Thus amino nitrogen production in cell-free extracts is markedly less sensitive to quinine and atabrine than is amino nitrogen formation in parasitized erythrocytes (cf. Tables V and VII).

It is of interest that crude papain activated with cyanide was completely insensitive to  $1 \times 10^{-3}$  M quinine and atabrine when tested under the conditions described in Fig. 1.

TABLE VII.

*Effect of Anaerobic Conditions and of Antimalarial Drugs upon Hydrolysis of Denatured Globin by Cell-Free Extracts*

The conditions were the same as in Table VI. Denatured horse globin was used as the substrate. The anaerobic samples were incubated in Thunberg tubes containing sticks of yellow phosphorus in the bulbs. The tubes were evacuated and refilled with nitrogen to atmospheric pressure. 2 hours, 40°.

Inhibitor	Inhibition of amino nitrogen formation
	<i>per cent</i>
Anaerobic conditions.....	3
Quinine, $1.0 \times 10^{-3}$ M.....	28
" $1.0 \times 10^{-4}$ ".....	3
" $1.0 \times 10^{-5}$ ".....	0
Atabrine, $1.0 \times 10^{-3}$ M.....	25
" $1.0 \times 10^{-4}$ ".....	3
" $1.0 \times 10^{-5}$ ".....	0

*Relation of Studies on Cell-Free Extracts to Problem of Oxidative Coupling—*

The lack of inhibition of protein hydrolysis in cell-free extracts under anaerobic conditions indicates that the effect of oxygen lack upon amino nitrogen formation in parasitized erythrocytes is not directly upon the proteinases involved. This constitutes direct evidence for the view that protein hydrolysis is coupled with oxidative reactions in the intact parasite. The difference in sensitivity of parasitized erythrocytes and cell-free extracts to quinine and atabrine also suggests the existence of such a coupling.

#### DISCUSSION

In the intact parasite, increase in amino nitrogen represents the difference between the rate at which the protein of the host is hydrolyzed and the rate at which new parasite protein is synthesized. Any factor which alters the

rate of either process will therefore change the amount of amino nitrogen which accumulates. Protein synthesis in the malaria parasite has not been studied, but it is probable that the inhibitory effect of anaerobiosis and of the antimalarials upon amino nitrogen formation is due to inhibition of protein hydrolysis, since it is unlikely that either anaerobiosis or the antimalarials would accelerate the rate of parasite protein synthesis. The stimulation of amino nitrogen formation produced by certain concentrations of quinine (Table V) may result from a differential effect upon the above two processes. If  $1 \times 10^{-4}$  M quinine inhibits the rate of parasite protein synthesis to a greater degree than it does the rate of host protein hydrolysis, then the observed increase in amino nitrogen accumulation might be produced.

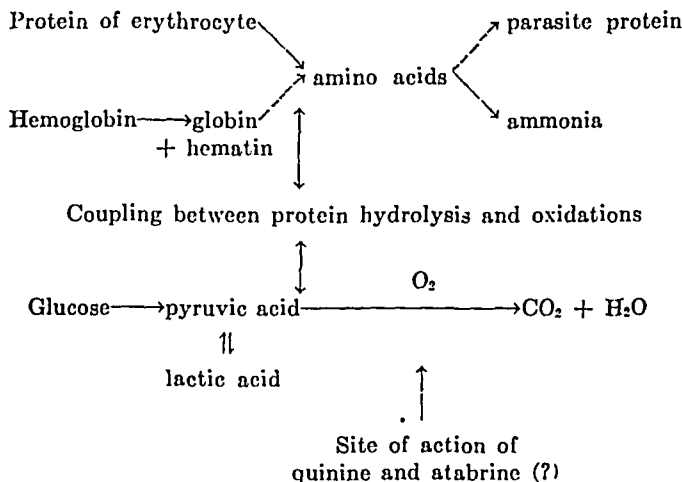
The linking of protein hydrolysis with oxidative reactions demands explanation, since the hydrolysis of peptide bonds is an exergonic process needing no such energetic coupling. However, oxidative energy may be needed for other processes intimately associated with hydrolysis of protein by the malaria parasite. For example, if hemoglobin does not penetrate the cell membrane of the parasite, the proteinases must then be secreted into the cytoplasm of the erythrocyte before hydrolysis occurs. Under these conditions, oxidative energy may be necessary for secretion of the proteinases into the erythrocyte. In homogeneous cell-free extracts, such a coupling would be unnecessary and none is observed. Several other similar explanations might equally well be offered.

Since oxidation of carbohydrate is a major process in the aerobic metabolism of the malaria parasite (13), and since oxidation of carbohydrate is sensitive to low levels of quinine and atabrine (13), it is logical to assume that protein hydrolysis in the intact parasite is coupled in some manner with carbohydrate oxidation. One observation difficult to reconcile with this assumption is that amino nitrogen production under aerobic conditions is not decreased in the absence of glucose from the medium. However, recent work (3), which indicates that the as yet unknown substance responsible for the respiration of parasitized erythrocytes in the absence of added substrate is oxidized over the same pathways as is glucose, makes this objection more apparent than real.

Although it is probable that hemoglobin is the protein hydrolyzed in parasitized erythrocytes, cell-free extracts of the malaria parasite hydrolyze hemoglobin at a slow and insignificant rate. In contrast, these extracts rapidly hydrolyze denatured globin. It is suggested as a tentative hypothesis that another enzyme system, present in intact parasites but absent from extracts, splits hemoglobin into heme and globin. Non-enzymic splitting of hemoglobin often results in the formation of denatured globin (16), and enzymic cleavage may also be accompanied by denaturation.

The denatured globin so formed may then be hydrolyzed by the enzymes present in the cell-free extracts and part of the liberated amino acids used for the synthesis of parasite protein.

The results of this investigation, together with the observations reported in other papers of this series (3, 4), may be summarized in a schematic representation of our present knowledge of the metabolism of the malaria parasite *Plasmodium gallinaceum* (reactions which have not been experimentally demonstrated are indicated by broken arrows).



#### SUMMARY

1. Chicken erythrocytes, parasitized with the malaria parasite *Plasmodium gallinaceum*, produce large amounts of amino nitrogen when incubated in air in the presence of glucose. When glucose is absent from the medium, much of the amino nitrogen appears as ammonia.

2. Normal chicken erythrocytes form only small amounts of either amino nitrogen or ammonia.

3. The production of amino nitrogen by parasitized erythrocytes is strongly inhibited by anaerobiosis and by low concentrations of quinine and atabrine.

4. Cell-free extracts of malaria parasites hydrolyze native hemoglobin at a very slow rate and split denatured globin at a much faster rate.

5. Production of amino nitrogen from denatured globin in cell-free extracts is not inhibited by anaerobic conditions and is inhibited only by relatively high concentrations of quinine and atabrine.

6. It is suggested that in the intact malaria parasite hydrolysis of protein is in some way linked to oxidative processes.

The infected chickens used in these experiments were kindly furnished by Mr. William Cantrell of the Department of Bacteriology and Parasitology.

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## AMINO ACID COMPOSITION OF SEED GLOBULINS

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Several years ago it was suggested that the crystalline globulins of the seeds of some of the Cucurbitaceae might provide a substitute for edestin for many types of work because of the doubtful availability of hemp-seed for future investigations (1, 2). With the use of the recently developed microbiological methods for amino acid analysis, it is now possible to extend our knowledge of the composition of these proteins. Estimations of the leucine, valine, and phenylalanine content have accordingly been made together with a reinvestigation of the tryptophane content. In this work, the globulins of pumpkin and squash (*Cucurbita moschata* and *Cucurbita maxima*), watermelon (*Citrullus vulgaris*), cucumber (*Cucumis sativus*), and, for comparison with these, edestin of hemp-seed and the crystalline globulin of tobacco seed have been studied. The proteins used in this investigation were all twice crystallized as described earlier, and in several instances were the identical preparations which had been analyzed previously (2). The data presented below are for the anhydrous ash-free proteins. All of these preparations are free of carbohydrate as determined by the orcinol reaction.

*Leucine, Valine, and Phenylalanine Determinations*—These amino acids were estimated by the microbiological method of Schweigert, McIntire, Elvehjem, and Strong (3) with *Lactobacillus arabinosus* 17-5. Hydrolysates were prepared by adding 40 cc. of 2 M HCl to 200 to 500 mg. of protein and autoclaving at 120° for 5 hours. The neutralized solutions were then assayed by comparison with standard amino acid growth tubes. For each hydrolysate, the average of several measurements at different growth levels is reported as a single determination. It was found earlier (4) that reliable estimations for these three amino acids were obtained by the above procedure, as judged by the agreement of our results and those of Brand (5) on human  $\gamma$ -globulin. The data are presented in Table I.

Our value for the leucine content of edestin is in close agreement with that of Brand, Ryan, and Diskant (6) who found 7.4 per cent and that of Fromageot and Mourgue (7) who obtained 7.31 per cent. The latter investigators also reported 5.7 per cent valine in edestin. Our results differ considerably from those obtained by Hegsted (8) who reported 5.4 per cent leucine, 4.2 per cent phenylalanine, and 4.7 per cent valine. Evidence

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that the latter determination is too low is also furnished by the work of Van Slyke and Levene (9) who actually isolated 5.6 per cent valine from this protein. By the Fischer ester method, Osborne and Liddle (10) obtained 3.09 per cent phenylalanine from edestin. Kapeller-Adler (11) found 3.9 per cent phenylalanine by a colorimetric method.

For the three amino acids recorded in Table I, there have been only a few analyses made on the cucurbit proteins. It is not without interest that in 1883 Schulze and Barbieri (12) isolated phenylalanine from squash seed globulin hydrolysate, thus providing the first demonstration that this amino acid is a protein constituent. Osborne and Clapp (13) isolated, by the ester method, 3.3 per cent phenylalanine and 0.3 per cent valine from a squash seed globulin. By a colorimetric method, Hess and Sullivan (14) found 5.8 per cent phenylalanine in squash seed globulin, a result

TABLE I  
*Leucine, Valine, and Phenylalanine Content of Seed Globulins*

Protein	Leucine		Valine		Phenylalanine	
	No. of determinations	Content	No. of determinations	Content	No. of determinations	Content
		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
Edestin..	4	7.43 $\pm$ 0.11	4	6.63 $\pm$ 0.12	4	5.45 $\pm$ 0.18
Pumpkin. . .	2	8.01 $\pm$ 0.00	4	6.50 $\pm$ 0.11	4	7.19 $\pm$ 0.08
Squash.....	2	7.99 $\pm$ 0.27	4	6.45 $\pm$ 0.25	4	6.76 $\pm$ 0.23
Watermelon...	2	7.53 $\pm$ 0.20	4	6.37 $\pm$ 0.11	4	7.70 $\pm$ 0.12
Cucumber... .	2	9.05 $\pm$ 0.00	2	6.96 $\pm$ 0.10	2	6.49 $\pm$ 0.11
Tobacco. .	2	10.5 $\pm$ 0.2	2	6.72 $\pm$ 0.16	2	5.70 $\pm$ 0.00

somewhat lower than that obtained by the microbiological assay. To the best of our knowledge, there have been no previous analyses of tobacco seed globulin for these amino acids.

*Tryptophane Content of Cucurbit and Tobacco Seed Globulins*—The tryptophane content of these proteins has previously been evaluated by both colorimetric and microbiological methods. However, since these earlier results were obtained on the hydrolyzed protein, it was of interest to compare these values with those obtained by the Bates method (15) on the unhydrolyzed protein. These determinations were performed exactly as described by Sullivan and Hess (16) by use of either a visual or a photoelectric colorimeter in conjunction with a filter having a broad transmission of about 525  $m\mu$  and comparison with tryptophane standards; the two methods gave identical results with samples of protein which contained about 0.5 mg. of tryptophane. It has already been shown that this method gives values in agreement with those obtained on hydrolyzed proteins by

either chemical methods (Sullivan and Hess (16)) or microbiological assays (Smith, Greene, and Bartner (4)).

The data presented in Table II show that the results obtained by the different methods of tryptophane determination are in good agreement, although the results obtained by the Folin-Marenzi method are generally lower. This is particularly true for the cucumber seed globulin in which the lowest value is probably erroneous, as judged by the agreement between the results of the other two methods. Sullivan and Hess found 1.69 per

TABLE II

*Tryptophane Content of Cucurbit and Tobacco Seed Globulins*

The numbers in parentheses refer to bibliographic citations.

Protein	Bates method (unhydrolyzed)	Microbiological (Ba(OH) <sub>2</sub> hydrolysis) method	Folin-Marenzi method
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Squash ( <i>Cucurbita</i> sp.).....	1.76 $\pm$ 0.06	1.76 (17)	1.69 (2), 1.71 (16)
Watermelon.....	1.90 $\pm$ 0.08	1.87 (17)	1.79 (2), 1.86 (18)
Cucumber.....	1.92 $\pm$ 0.08	1.94 (17)	1.71 (2)
Tobacco.....	1.51 $\pm$ 0.04	1.49 (17)	1.41 (2)

TABLE III

*Tryptophane Content of Edestin*

Preparation	Microbiological method	Bates method
	<i>per cent</i>	<i>per cent</i>
A	1.28 $\pm$ 0.04	1.21 $\pm$ 0.03
B	1.25 $\pm$ 0.02	1.23 $\pm$ 0.02
C	1.24 $\pm$ 0.04	1.23 $\pm$ 0.02

cent tryptophane for squash seed globulin by the Bates method on the unhydrolyzed protein.

*Tryptophane Content of Edestin*—This protein has been repeatedly analyzed for tryptophane with the Folin procedures and their modifications, and consistent values of 1.45 to 1.52 per cent have been obtained by many different investigators (19, 2). However, we have found that, by microbiological assay and by estimation with the Bates method, the tryptophane content of edestin is 1.24 per cent. The colorimetric procedures were performed on the unhydrolyzed protein, as described above. The microbiological assays were performed by the procedure of Greene and Black (17) with *Lactobacillus arabinosus* 17-5 on protein hydrolyzed by Ba(OH)<sub>2</sub>; the values obtained were multiplied by 2, assuming complete racemization during the hydrolysis. The results are given in Table III.



Each of the microbiological values given is the average of at least four separate determinations; *i.e.*, independent hydrolyses tested at several different growth levels. Likewise, each of the results given for the Bates method represents many determinations mostly performed with the photo-electric colorimeter. We are indebted to Dr. H. B. Vickery for supplying us with two of the preparations used: Preparation A which was a mixture of several preparations made by the late T. B. Osborne and dated 1914, and Preparation B which was prepared in 1928 and is the same preparation which had been analyzed for arginine (Vickery (20)) and histidine (Vickery and Winternitz (21)). Preparation C was made by one of us (E. L. S.) in 1940 in Dr. Vickery's laboratory and contained 1.45 per cent tryptophane according to the Folin and Marenzi method (2).

The results in Table III for the three different preparations clearly show that the two methods newly applied to edestin yield identical results which are considerably lower than those found by the older colorimetric methods of Folin and his collaborators.

#### DISCUSSION

The tryptophane content of edestin has always played a prominent part in estimations of its minimum molecular weight. The most recent discussion of the composition of edestin is given by Chibnall (22) who deduces from both physical measurements and the available analytical data a minimum unit of about 50,000 which is one-sixth the actual molecular weight. However, his use of 1.50 per cent tryptophane involves an error of about 12 per cent on the basis of his calculation of 4 residues for this unit. Our average value of 1.24 per cent is consistent with a content of 3 tryptophane residues, leading to a calculated unit weight of 49,400. As for the other three amino acids for which values are reported in this paper, edestin contains 16 phenylalanine residues (calculated unit = 48,500), and 28 residues each of leucine and valine (calculated unit = 49,600).

In the absence of physical measurements, it is futile to discuss in detail the composition of the other seed globulins. However, it is noteworthy that, on the basis of tryptophane content alone, the unit weights for these proteins must differ somewhat from that of edestin and from each other. While determinations of the other amino acids are of little value at the present time for interpretation of the molecular size, they show certain interesting relationships. The valine figures are very similar for all of the globulins, even those of widely different botanical origin. On the other hand, the leucine and phenylalanine contents of cucumber seed globulin clearly distinguish this protein from the others derived from the same family. In fact, the proteins from all the different genera are easily recognized as being different in composition. The two species of *Cucurbita*

are obviously identical in leucine and valine, but show a small though possibly doubtful difference in phenylalanine content.

#### SUMMARY

The crystalline seed globulins of hemp (edestin), tobacco, and several species of Cucurbitaceae (squash, pumpkin, watermelon, and cucumber) have been analyzed for leucine, phenylalanine, and valine by microbiological assay with *Lactobacillus arabinosus* 17-5. The cucurbit proteins and tobacco seed globulin are shown to yield tryptophane values, by a photometric method on the unhydrolyzed protein, which are in agreement with earlier results obtained by microbiological and colorimetric methods on the hydrolyzed proteins. On the other hand, by the newer methods of assay the tryptophane content of edestin is somewhat lower than that found earlier by the Folin-Marenzi and similar methods.

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# CONVERSION OF SULFIDE SULFUR TO CYSTINE SULFUR IN THE RAT, WITH USE OF RADIOACTIVE SULFUR\*

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Shortly after radioactive sulfur ( $S^{35}$ ) became available, Tarver and Schmidt definitely established that the sulfur of methionine could be used in the synthesis of cystine *in vivo* (13). Du Vigneaud *et al.* (15) have confirmed the above, using the stable isotope  $S^{34}$ , and have at the same time shown that the  $\beta$ - and  $\gamma$ -carbon atoms of methionine are not used in the synthesis of cystine. That serine may furnish the carbon chain for the synthesis of cystine *in vivo* has been demonstrated by Stetten (12) and by Binkley and du Vigneaud (3). From studies on the thio ether, cystathionine, in the intact animal and with tissue slices, evidence is at hand to indicate that cystathionine, formed from homocysteine and serine, may split to form cysteine (2, 14).

That another mechanism may also be available for the synthesis of cystine in the animal organism is suggested by the observation of Smythe and Halliday that the enzyme preparation which can convert cysteine to pyruvic acid, ammonia, and hydrogen sulfide can, *in vitro*, promote incorporation of sulfide sulfur into cysteine (10). That this enzyme, cysteine desulfurase, is different from the enzyme effective in promoting the transfer of the sulfur of methionine or homocysteine to serine is demonstrated by an observation of Binkley and du Vigneaud (3) that the former is inhibited by 0.01 M cyanide, whereas the latter is not.

In experiments on the distribution of sulfur in various tissues of the rat after the oral administration of sodium sulfide containing radioactive sulfur ( $S^{35}$ ), it was noted that all the tissues examined contained some of the radioactive sulfur (4). In view of the *in vitro* demonstration of Smythe and Halliday (10) it was of interest to see whether the cystine of the hair and other tissues of the animals employed had any of the radioactive isotope. This appeared to be a remote possibility since the animal tissues had been removed 24 hours after a single oral administration of the sulfide sulfur, which was for the most part excreted by the end of this period of time. It was a surprise, therefore, to find on isolation of cystine from hair and muscles of the animals that the cystine from each source had a slight but definite concentration of radioactive sulfur. The experiments herein reported are an extension of this initial observation.

\* Aided by a grant from the Bristol-Myers Company.

## EXPERIMENTAL

From irradiated carbon tetrachloride<sup>1</sup> radioactive sulfur ( $S^{35}$ ) was isolated as barium sulfate by the method proposed by Kamen (7). The barium sulfate was subsequently reduced to barium sulfide with a mixture of aluminum, carbon, and sodium bicarbonate (9). Hydrogen sulfide produced from the barium sulfide by the action of dilute hydrochloric acid was transferred by a current of nitrogen gas into a neutral solution of cadmium sulfate. From the latter solution the cadmium sulfide formed was isolated by centrifugation in a previously weighed 15 ml. conical centrifuge tube. After repeated washing with distilled water the cadmium sulfide and tube were dried to constant weight at  $110^{\circ}$ . Hydrogen sulfide was released from the cadmium sulfide by treatment with hydrochloric acid and the hydrogen sulfide was transferred by a stream of nitrogen to a dilute solution of sodium hydroxide calculated to neutralize exactly the amount of hydrogen sulfide so formed. To the solution of sodium sulfide containing the radioactive sulfur, an additional amount of inactive sodium sulfide in solution was added and the whole diluted to a volume such that each ml. contained approximately a mg. of sulfide sulfur.

The activity of the sulfide sulfur was determined by oxidizing a suitable portion of the sulfide sulfur with a mixture of sodium carbonate and sodium peroxide (1) and isolating the resultant sulfate sulfur as barium sulfate. This was then counted as previously described (4). Counting was repeated at intervals of a few days to a few weeks. From the repeated counts the calculated rate of disintegration indicated a half life of approximately 88 days. This half life is the one assigned to  $S^{35}$  (7).

Two young albino rats (Rat A, male, 164 gm.; Rat B, female, 127 gm.) were kept in metabolism cages to allow collection of urine. They were fed for only 30 minutes in separate cages at the beginning of each 24 hour period. Immediately after the daily feeding period each rat received by stomach tube 0.15 ml. of bromobenzene (Merck, reagent grade) for 3 consecutive days. 30 minutes later 0.5 ml. of a solution of sodium sulfide containing radioactive sulfur was injected intraperitoneally. A similar injection was given approximately 6 hours later. The injections were continued, each rat receiving a mg. of sulfide sulfur daily, until each animal had been given approximately 11 mg. of sulfide sulfur and a total of  $10.92 \times 10^5$  counts per minute of  $S^{35}$ . The rats were then sacrificed and cystine isolated from their hair, skin, muscle, and liver. After the 4th day of the experiment Purina dog biscuits were continuously accessible to the rats.

Mercapturic acid was isolated by a method essentially that proposed

<sup>1</sup> The carbon tetrachloride was kindly supplied by Dr. M. D. Kamen, Washington University, St. Louis, Missouri.

by McGuinn and Sherwin (8). The urine was collected in 24 hour periods, pooled, acidified with concentrated hydrochloric acid until definitely acid to Congo red paper, and extracted with chloroform in a continuous extractor for 8 to 12 hours. The chloroform was removed by distillation *in vacuo*. The residue in the distilling flask was dissolved in dilute ammonium hydroxide, decolorized with norit, and then acidified with hydrochloric acid. Upon acidification there occurred an immediate precipitation of mercapturic acid. Crystallization was allowed to continue overnight in a refrigerator. The mercapturic acid was then isolated by centrifugation. It was washed twice with distilled water, again dissolved in dilute ammonium hydroxide, and precipitated by the addition of hydrochloric acid. Recrystallization of the mercapturic acid was repeated four times in this manner. Finally, the product, which at this point was crystalline and white, was isolated on a Hirsch funnel. It was washed repeatedly with distilled water and dried for a few days *in vacuo* over calcium chloride. The yield was 15 mg.; consequently only the melting point and radioactivity were determined. This is listed as Sample 1 in Table I.

In view of the fact that two stock rats, weighing approximately 200 gm., which had received the above quantity of bromobenzene alone, produced enough mercapturic acid to allow for the isolation of 188 mg. of the material, the experiment was repeated. Two additional male albino rats (Rat C, 254 gm.; Rat D, 250 gm.) were each given a total of 6.5 mg. of sulfide sulfur containing radioactive sulfur at the rate of a mg. each day before they were sacrificed and their tissue taken for cystine isolation. The total activity of  $S^{35}$  given each was  $33.65 \times 10^4$  counts per minute. For the first 3 days of the experimental period they received 0.15 ml. of bromobenzene daily. From the urines of Rats C and D a total of 225 mg. of mercapturic acid was isolated (Sample 2 in Table I).

Sample 3 of Table I represents mercapturic acid isolated by the same procedure from the pooled urine of two rats that together received 0.8 ml. of bromobenzene and 1.5 mg. of sulfide sulfur containing a total of  $11.32 \times 10^4$  counts per minute of radioactive sulfur. The materials were given in divided doses over a period of 3 days. The yield of mercapturic acid was 102 mg.

Sample 4 of Table I is a sample of mercapturic acid originally isolated from the urine of rats receiving only bromobenzene. It was added to urine of rats that had received by intraperitoneal injection only a solution of sodium sulfide containing radioactive sulfur. It was recovered from the latter urine by the procedure used in the isolation of the mercapturic acid samples already mentioned. This experiment was designed to determine the extent of possible contamination of mercapturic acid by sulfur in

all the forms present in rat urine after sodium sulfide administration. The urine aliquot used had a total activity of  $10.5 \times 10^4$  counts per minute.

Cystine was isolated as described by Tarver and Schmidt (13) from the various tissues of Rats A, B, and C. It was recrystallized an additional three times by solution in 6 N hydrochloric acid, followed by adjustment with 8 N sodium acetate to approximately pH 4. The samples were dried over calcium chloride. The same procedure was used in isolating cystine from the hair of Rats E and F. The last two rats had not received any radioactive sulfur. Sodium sulfate, however, containing radioactive sulfur was added to the hair prior to hydrolysis with hydrochloric acid. Each hair sample was thus contaminated with sulfate sulfur containing

TABLE I

*Specific Activities of Mercapturic Acid Isolated from Urine of Rats Given Bromobenzene Orally and Sodium Sulfide Containing Radioactive Sulfur ( $S^{35}$ ) by Intraperitoneal Injection*

Sample No.	Found*			
	M.p.	Nitrogen	Sulfur	Counts per min. per mg. S
	°C.	per cent	per cent	
1	152-154			$11.7 \pm 2$
2	152-154	4.41	10.09	$7.0 \pm 1$
3	152-153	4.45	9.99	$3.6 \pm 0.5$
4†	152-154			$2.5 \pm 0.2$

\* Calculated, N 4.40, S 10.06. Reported melting point,  $152^\circ$  (11).

† Mercapturic acid recovered from urine contaminated with radioactive sulfur.

radioactive sulfur with an activity of  $25.0 \times 10^3$  counts per minute. Isolation of cystine from the tissues of Rat D was carried out only to the cuprous mercapturic stage.

In most cases a sufficient amount of cystine and mercapturic acid was isolated to allow for a determination of sulfur content and melting point as well as radioactivity. Sulfur was determined by sodium peroxide fusion of the samples in a Parr bomb. In a few cases nitrogen was determined by a micro-Kjeldahl procedure. A complete analysis on two samples of mercapturic acid is given in Table I. Typical of the results obtained when cystine samples were analyzed are the results on the cystine from the skin of Rat C: melting point,  $258-259^\circ$  with decomposition (uncorrected); N, 11.62 per cent; S, 26.80 per cent. The calculated values are N 11.65 per cent, S 26.68 per cent. The reported melting point is  $258-261^\circ$  with decomposition (6).

For radioactivity measurements the sulfur of mercapturic acid or cystine

was changed to sulfate sulfur by sodium peroxide fusion in a Parr bomb. The barium sulfate formed on the addition of barium chloride was isolated by centrifugation and deposited in an even layer in counting cups (4, 5). To eliminate differences in self-absorption, the amount of barium sulfate used in each case was the same. This was accomplished by taking samples of 25 mg. of cystine or a weight of mercapturic acid which would give an equivalent weight of sulfur. In case the isolated samples were not large enough, ordinary cystine was added to make up the difference before peroxide fusion.

The relative concentration of radioactive sulfur in the barium sulfate samples was determined with a Geiger-Müller, bell type counter (4). At the time samples obtained in any one experiment were counted an aliquot of the radioactive sulfide sulfur solution used was likewise counted.

TABLE II

*Specific Activities of Cystine Isolated from Rat Tissue after Intraperitoneal Injection of Sodium Sulfide Containing Radioactive Sulfur ( $S^{35}$ )*

Source of cystine	Counts per min. per mg. sulfur					
	Rat A	Rat B	Rat C	Rat D	Rat E	Rat F
Hair.....	50 $\pm$ 5	86 $\pm$ 8	3 $\pm$ 0.5	4 $\pm$ 0.4	1 $\pm$ 1*	0.5 $\pm$ 1*
Skin.....	22 $\pm$ 2	17 $\pm$ 2	5 $\pm$ 0.5	25 $\pm$ 3		
Muscle.....	61 $\pm$ 6	166 $\pm$ 15	4 $\pm$ 0.2	13 $\pm$ 2		
Liver.....	16 $\pm$ 1	17 $\pm$ 2	2 $\pm$ 0.2	8 $\pm$ 1		

\* Cystine recovered from hair of stock animals. Sodium sulfate containing radioactive sulfur was added to the hair before isolation of the cystine was initiated.

The specific activities of the cystine samples isolated are given in Table II.

#### DISCUSSION

An examination of the specific activities of the mercapturic acid and cystine samples isolated, as given in Tables I and II, after the administration of sodium sulfide containing radioactive sulfur, shows that some of the sulfide sulfur was incorporated into each. The amount of radioactive sulfur found in the samples is not large, but even in those samples in which it is smallest it is slightly greater than that attributable to contamination by radioactive sulfur present in a form other than cystine or mercapturic acid sulfur. This observation is a substantiation *in vivo* of the observation of Smythe and Halliday (10) that hydrogen sulfide can be enzymatically incorporated into cysteine *in vitro*. The amount of sulfide sulfur thus used, however, in the intact animal appears to be small and in some cases



practically negligible. It was hoped that by a preliminary feeding of bromobenzene some of the cystine of the body would be employed in the synthesis of mercapturic acid and that subsequently a restoration of the cystine stores would occur when bromobenzene was discontinued and food was freely accessible. It appeared reasonable to expect synthesis of cystine to occur as an aid in the restoration process. That depletion of protein stores did occur seemed to be indicated by a marked loss of weight of the animals during bromobenzene feeding. This loss was in part restored when bromobenzene was discontinued. It may be that most of the cystine of the restored protein, if gain in weight here can be assumed as indicative of such a process, came from preformed cystine of the food and synthesis from methionine and serine (2, 3, 14) found in the food, with only a negligible amount being added by a process employing sulfide sulfur. It may be tentatively concluded that normally, on the basis of the observations reported here and in view of the fact that administered sulfide sulfur is rapidly, and to a large extent, excreted as sulfate sulfur, the mechanism whereby cystine may be synthesized *in vivo* from sulfide sulfur accounts for only a very small amount of the cystine in the proteins of the rat tissues analyzed.

#### SUMMARY

Sulfide sulfur can be used by the intact rat for the synthesis of cystine as indicated by the use of radioactive sulfur ( $S^{35}$ ). The radioactive sulfur from sulfide was found incorporated in the mercapturic acid synthesized after bromobenzene administration and was also present in the cystine isolated from hair, liver, skeletal muscle, and skin.

The amount of cystine synthesized by the process employing sulfide sulfur was found to be small, however, even though there was an active deposition of protein as indicated by an increase in body weight.

The continuing interest and advice of Professor C. S. Robinson are gratefully acknowledged. It is likewise a pleasure to thank Dr. C. W. Sheppard and Dr. P. F. Hahn for their interest and critical discussion of questions which arose in the course of the work.

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# THE INFLUENCE OF CERTAIN SUBSTANCES ON THE ANTIBIOTIC ACTIVITY OF STREPTOMYCIN IN VITRO

## II. THE ACTION OF SOME CARBONYL REAGENTS ON STREPTOMYCIN

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Recently Brink, Kuehl, and Folkers (1) reported that streptomycin is inactivated by certain carbonyl reagents and adduced evidence tending to show that this inactivation is due to distinct chemical combination between these reagents and a carbonyl group in streptomycin.

During the past few months a detailed investigation of this type of inactivation has been in progress in this laboratory. Our objective has been (a) to ascertain which chemical grouping in streptomycin is involved and (b) to examine the antibacterial properties of mixtures containing the inactivation products in equilibrium with their parent components.

There is little doubt from our results that streptomycin contains a carbonyl group capable of reacting with great rapidity with such reagents as semicarbazide, hydroxylamine, and phenylhydrazine to form a semicarbazone, oxime, and phenylhydrazone. Among derivatives of this kind, the thiosemicarbazones are particularly useful, inasmuch as they can be recognized in solution by their intense absorption bands in the ultraviolet region (2). Absorption curves<sup>1</sup> of the thiosemicarbazones of acetone, inosose (2,3,4,5,6-pentahydroxycyclohexanone) (3), and streptomycin as well as of thiosemicarbazide alone, in water, are given in Fig. 1. The curve for streptomycin itself is not included in this figure, since it shows only slight end-absorption. It can be seen that the spectrum of acetone thiosemicarbazone reaches a maximum of high intensity ( $\epsilon = 19,000$ ) at 263  $m\mu$ . Substitution of hydroxyl groups in the  $\alpha, \alpha'$  positions of the carbonyl group, as in inosose, displaces this band to 269  $m\mu$  ( $\epsilon = 25,400$ ).

When 1 mole of streptomycin trihydrochloride and 1.5 moles of thiosemicarbazide are dissolved in water and the ultraviolet spectrum of the mixture determined after 15 hours standing at room temperature, a band with a maximum at 270  $m\mu$  ( $\epsilon = 22,000$ ) is found.<sup>2</sup> The position and intensity of this band show convincingly that the light-absorbing entity is a

<sup>1</sup> We are indebted to Dr. N. H. Coy of the Squibb Biological Laboratories for the absorption spectra reported in this paper.

<sup>2</sup> The maxima of the three thiosemicarbazones shown in Fig. 1 are displaced by 4 to 5  $m\mu$  towards longer wave-lengths, if the solvent is methanol instead of water.

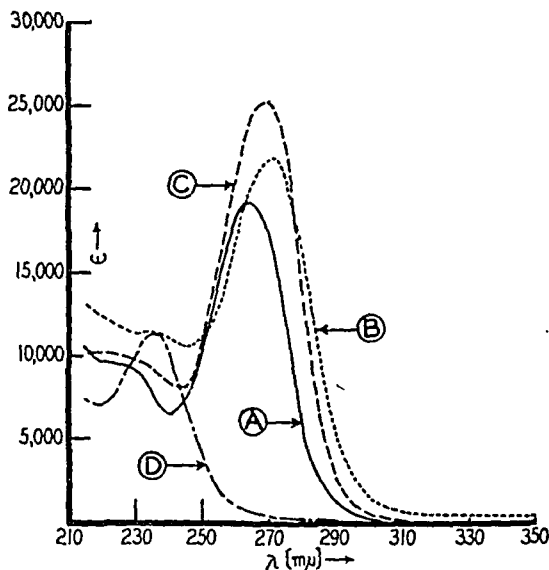


FIG. 1. Ultraviolet absorption spectra (solvent, water): Curve A, acetone thiosemicarbazone; Curve B, streptomycin + thiosemicarbazide after standing for 15 hours; Curve C, inosose thiosemicarbazone; Curve D, thiosemicarbazide.

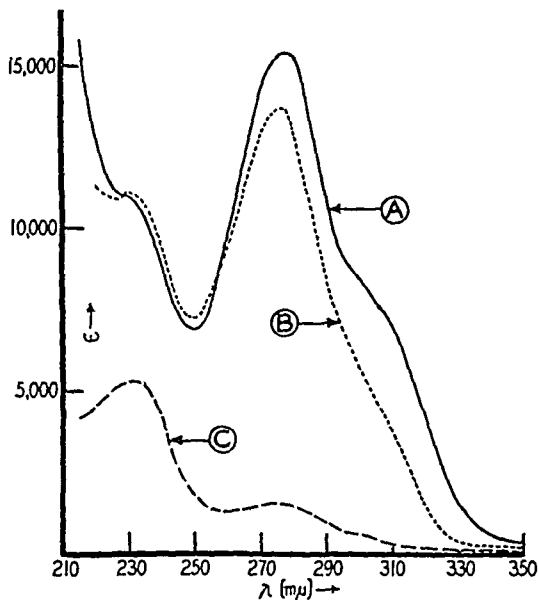


FIG. 2. Ultraviolet absorption spectra (solvent, water): Curve A, streptomycin + phenylhydrazine; Curve B, inosose + phenylhydrazine; Curve C, phenylhydrazine. All spectra taken after standing for 2 hours in the presence of a small amount of acetic acid.

thiosemicarbazone, formed by the action of thiosemicarbazide on a carbonyl group in streptomycin. Furthermore, the close proximity of the maxima given by streptomycin and inosose thiosemicarbazones suggests that this carbonyl group is part of a carbohydrate-like moiety. The ease with which the reaction proceeds may be taken as an indication that the reactive carbonyl group is present in streptomycin as such and not in combined form.

Comparison of the absorption spectra of the phenylhydrazones (4) of streptomycin and inosose leads to similar conclusions (Fig. 2).

The study of the antibacterial properties of the reaction products of streptomycin and the above carbonyl reagents is complicated by several factors. The germicidal action of the carbonyl reagents has long been known (5). Hence, in assaying streptomycin-carbonyl reagent mixtures, it is important to know whether any residual activity is caused by excess reagent, by excess streptomycin, or by the respective streptomycin derivative formed. Further, the addition to assay media of certain substances which do not inactivate streptomycin may nevertheless interfere with its bacteriostatic action (6).

In the present work two test media were employed, (a) 0.75 per cent tryptone broth and (b) an enriched thioglycolate broth (of the same composition as that used in earlier work) (6). *Klebsiella pneumoniae* was used as the test organism.

### Procedure

To 2 ml. of solution containing 68.8 mg. of streptomycin (in terms of free streptomycin base<sup>3</sup>) were added 2 ml. of a given concentration of the carbonyl reagent being tested. The carbonyl reagent solutions were adjusted to pH 5.3, with 50 per cent potassium acetate solution for the adjustment of hydrochlorides of semicarbazide and hydroxylamine, and acetic acid for thiosemicarbazide and hydrazine hydrate. The latter compound was also tested at pH 8.5.

The streptomycin-carbonyl reagent mixtures were incubated at 25° for 20 hours. Then, with a broth dilution assay procedure (7), the minimum volume was determined of each mixture which, when added to 2 ml of test broth, caused complete inhibition of growth of *Klebsiella pneumoniae* under standard conditions. Appropriate controls of streptomycin and carbonyl reagent, respectively, at pH 5.3 (and at pH 8.5 in the one case mentioned above) were included in all experiments.

<sup>3</sup> The streptomycin preparation used for this work contained 300  $\gamma$  of free streptomycin base per mg. of total solids. Throughout this paper streptomycin concentrations are given in micrograms per ml. based on the definition of the Food and Drug Administration that 1 unit equals 1  $\gamma$  of free streptomycin base.

TABLE I  
Inactivation of Streptomycin with Semicarbazide at pH 5.3

Mixture No.		Composition of mixture,* NH <sub>2</sub> CONHNH <sub>2</sub> ·HCl per γ streptomycin	At end-point concentration†												Ratio† $\frac{E'}{E}$
			In tryptone broth						In thioglycolate broth						
			Total concentration streptomycin γ per ml. (A)	Total concentration NH <sub>2</sub> CONHNH <sub>2</sub> ·HCl γ per ml. (B)	$\frac{A}{J}$ (C)	$\frac{B}{K}$ (D)	C + D γ (E)	Inacti- vation (F) per cent	Total concentration streptomycin γ per ml. (A')	Total concentration NH <sub>2</sub> CONHNH <sub>2</sub> ·HCl γ per ml. (B')	$\frac{A'}{J'}$ (C')	$\frac{B'}{K'}$ (D')	C' + D' γ (E')	Inacti- vation (F') per cent	
[S-K <sub>1</sub>		γ NH <sub>2</sub> CONHNH <sub>2</sub> ·HCl control													1.0
S-1	8.72	0.790	6.90	14.4	0.10	14.5	93.1	28.1	249	20.1	0.9	21.0	95.2	1.45	
S-2	5.81	0.695	4.08	12.6	0.06	12.7	92.1	36.6	215	26.2	0.8	27.0	96.3	2.12	
S-3	2.91	0.520	1.52	9.45	0.02	9.47	89.4	58.5	170	41.7	0.6	42.3	97.6	4.46	
S-J <sub>1</sub>	Streptomycin control	0.055		1.0		1.0		1.40		1.0		1.0		1.0	
S-K <sub>2</sub>	NH <sub>2</sub> CONHNH <sub>2</sub> ·HCl control		65.5		1.0	1.0			238		1.0	1.0		1.0	
S-4	2.91	0.680	1.97	12.4	0.03	12.4	91.9	55.5	161	51.4	0.68	52.1	98.1	4.21	
S-5	1.45	0.615	0.89	11.2	Negligi- ble	11.2	91.1	50.0	73.0	46.3	0.31	46.6	97.9	4.16	
S-6	0.725	0.715	0.52	13.0	"	13.0	92.3	40.8	30.0	37.8	0.13	37.9	97.4	2.92	
S-7	0.363	0.685	0.25	12.5	"	12.5	92.0	21.9	8.0	20.3	0.03	20.3	95.1	1.62	
S-8	0.182	0.219	0.04	3.98	"	4.0	75.0	4.2	7.6	3.9	0.03	3.9	74.0	0.98	
S-J <sub>2</sub>	Streptomycin control	0.055		1.0		1.0		1.08		1.0		1.0		1.0	

- \* The work with semicarbazide was done in two separate experiments. Hence there are two sets of streptomycin and semicarbazide controls.
- † Concentration in the broth containing the minimum inhibiting volume of the given reaction mixture.
- ‡ See the text.
- § Ratio of total streptomycin concentration at the end-point, when tested as streptomycin-carbonyl reagent mixture, to the end-point concentration of the streptomycin control. (Value in column *A*, for given mixture, divided by value of streptomycin control in the same column.)
- || Ratio of total carbonyl reagent concentration at the end-point, when tested as streptomycin-carbonyl reagent mixture, to the end-point concentration of carbonyl reagent control. (Value in column *B*, for given mixture, divided by value of carbonyl reagent control in the same column.)
- ¶ Sum of ratio in column *C* plus that in column *D* for the given mixture.



From the minimum inhibiting volumes of reaction mixtures thus determined, it was possible to calculate total concentrations of parent substances (i.e., streptomycin and carbonyl reagent) present in the broths at the end-point. These total concentrations include, of course, both combined and free streptomycin and carbonyl reagent respectively.

TABLE II  
*Inactivation of Streptomycin with Various Carbonyl Reagents at pH 5.3*

	Composition of mixture, carbonyl reagent per microgram streptomycin	Inactivation as assayed in		Ratio $\frac{E'}{E}$
		Tryptone broth	Thioglycolate broth	
	$\gamma$	per cent	per cent	
Thiosemicarbazide	Control			1.0
	1.16	92.9	97.1	2.46
	0.580	92.8	95.6	1.62
	0.290	87.6	91.6	1.46
	0.073	35.0	37.5	1.03
Hydroxylamine hydrochloride	Control			1.0
	2.92	96.9	96.0	0.78
	1.46	97.3	97.1	0.92
	0.730	97.4	97.6	1.09
	0.365	96.3	97.4	1.46
Hydrazine hydrate	0.183	95.2	97.3	1.76
	Control			1.0
	3.34	†	70.0	3.40
	1.67	37.0	73.0	2.6
	0.835	†	58.0	2.7
Streptomycin	0.418	38.0	47.0	1.4
	0.209	†	41.0	2.0
	Control			1.0

\* See the text.

† The activity of the mixture when tested in tryptone broth was slightly greater than that of the streptomycin and hydrazine hydrate controls combined.

As already indicated, detailed studies were carried out with semicarbazide hydrochloride, hydroxylamine hydrochloride, thiosemicarbazide, and hydrazine hydrate. Since the procedure for analysis of the results was similar for all four compounds, complete data are presented for only semicarbazide (Table I), whereas the results with the other three compounds are summarized in Table II.

In Table I the total end-point concentrations of streptomycin in a given broth are listed in column *A*, and of semicarbazide hydrochloride in column *B*. In column *C* are listed the ratios of total end-point concentration

of streptomycin, when tested in the form of streptomycin-carbonyl reagent mixture, to the end-point concentration of the streptomycin control. Similarly in column *D* are given the ratios of total end-point concentrations of carbonyl reagent, when tested in mixture with streptomycin, to the end-point concentration of the carbonyl reagent control. The sum of the ratios listed in columns *C* and *D* for each mixture are listed in column *E*.

It will be noted that the end-point concentration of the streptomycin controls in 0.75 per cent tryptone broth in all experiments was 0.055  $\gamma$  per ml., while in thioglycolate broth this figure varied with the experiment. In a broth prepared from a given lot of tryptone at a fixed concentration, the end-point concentration for streptomycin was found to be remarkably constant, while in thioglycolate broth it varied with the age of the broth (6). Hence, an average figure was used for the calculation of the end-point concentrations of streptomycin in tryptone broth. When thioglycolate broth was used as the test medium, it was necessary to complete all assays for a given experiment in a given lot of broth within a few days in order to obtain end-point concentrations which did not vary by more than 10 to 15 per cent. Thus each experiment in this broth had its own end-point concentration for the streptomycin and carbonyl reagent controls, respectively.

It will be recognized on examination of Table I that the figures in column *C* represent how many times more streptomycin was needed to cause inhibition, when added in mixture with the carbonyl reagent, than was required of the streptomycin control (or, in other words, an index of the degree of inactivation of the streptomycin). The ratios in column *D*, on the other hand, never exceed 1.0, thus indicating that the end-point concentrations of carbonyl reagent, resulting from titrating the carbonyl reagent-streptomycin mixtures, were never as high as the inhibiting concentrations as determined by the carbonyl reagent controls. The closer these ratios approached 1.0, the greater was the rôle played by the carbonyl reagent, *per se*, in inhibiting the growth of the test organism. In the tryptone broth the carbonyl reagents played little rôle and the maximum ratio for thiosemicarbazide was 0.016, for hydrazine hydrate 0.035, for semicarbazide 0.10, and for hydroxylamine 0.78. In thioglycolate broth, where the activity of streptomycin and possibly that of its semicarbazone, etc., are greatly lowered, the carbonyl reagents played a larger rôle in the final inhibition, and the maximum figures were 0.9 for thiosemicarbazide and for semicarbazide, 0.82 for hydroxylamine, and 0.51 for hydrazine hydrate.

It should be pointed out that semicarbazide, which is one of the least bactericidal carbonyl reagents tested (8), was studied at higher concentrations than any of the others. The maximum concentration of thiosemicarbazide which could be used in the tests was limited by the relative insolubility of this compound in water.

It is of interest to note that, while thiosemicarbazide inhibits bacterial growth at lower concentrations in thioglycolate than in tryptone broth, the reverse is true for the other carbonyl reagents tested.<sup>4</sup>

*Significance of  $E'/E$  Ratios*—If the minimum inhibiting volume of a bactericidal or bacteriostatic substance is added to a culture medium, it might be said that the culture medium contains 1 inhibiting dose per ml. If a mixture of inhibitory substances is added, as was the case in this work, the net effect of adding the minimum inhibiting volume of such a mixture is still that of 1 inhibiting dose. From the stand-point of the growth-inhibiting activities of free streptomycin and free carbonyl reagent, respectively, the addition of the end-point volume of the various reaction mixtures would have resulted in concentrations much higher than 1 inhibiting dose per ml. in each broth if no reaction had occurred first between the two parent substances in the mixture. Since the given volume of each mixture had, in fact, only the effect of 1 inhibiting dose, the parent substances had retained only a fraction of their original antibacterial activities. The inverse of the figure in column  $E$  in Table I for each mixture is a measure of this residual activity in a given broth.

Thus the figures in column  $E$  for each parent substance (*i.e.*, streptomycin and carbonyl reagent control solutions) are always equal to 1.0. The ratio  $E'/E$  in the two broths for either parent substance is, then, also equal to 1.0. On the other hand, when we study these ratios for the various streptomycin-carbonyl reagent mixtures, we find that these values approach 1.0 only when one or the other parent substance is present in excess in the mixture, and otherwise are greater than 1.0. This is best exemplified in Table I, where this ratio rises to approximately 4.5 for semicarbazide.

That these ratios for the reaction mixtures exceed 1.0 may indicate that the reaction compounds have antibacterial activities of their own which are affected differently by the two test broths than are the activities of either parent substance. Thus while thioglycolate broth interferes more than does tryptone broth with the bacteriostatic activities of both streptomycin and semicarbazide, it (thioglycolate broth) causes even greater interference with the apparent activity of the semicarbazone. This may be an effect on the semicarbazone activity itself or may, perhaps, result from greater dissociation of the semicarbazone, releasing more streptomycin in tryptone broth than in thioglycolate broth. The answer to this question must await an accurate study of the equilibria involving streptomycin, the carbonyl reagents, and the corresponding reaction products.

<sup>4</sup> The minimum inhibiting concentrations in tryptone broth and thioglycolate broth, respectively, were as follows in micrograms per ml.: hydrazine hydrate 5.0 and 41.4; hydroxylamine hydrochloride 6.5 and 147; semicarbazide hydrochloride 66.0 and 275; and thiosemicarbazide 76.5 and 38.0.

Of the carbonyl reagents studied, hydrazine hydrate is the least effective in inactivating streptomycin. As indicated above, this reaction was studied both at pH 5.3 and 8.5. Since similar results were obtained in both studies, data obtained only at pH 5.3 are presented.

#### SUMMARY

1. Evidence is presented to show that the inactivation of streptomycin by carbonyl reagents is due to the presence in streptomycin of a ketone or aldehyde group which forms part of a carbohydrate-like moiety.

2. The absorption spectra of the thiosemicarbazone and phenylhydrazone of streptomycin are given.

3. Over 90 per cent inactivation of streptomycin occurs at room temperature when 1  $\gamma$  of streptomycin is mixed with as little as 0.2 to 0.3  $\gamma$  of certain carbonyl reagents.

4. Inactivations up to 98 per cent of the streptomycin activity have been demonstrated without significant interference in the bioassays by the bacteriostatic properties of the carbonyl reagents.

5. Attempts to attain higher degrees of inactivation by increasing carbonyl reagent concentrations are complicated by antibacterial activity attributable to the carbonyl reagents.

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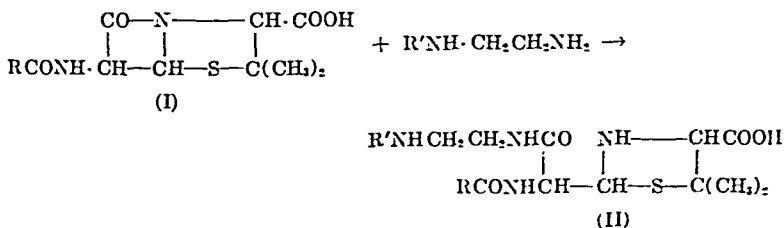
# A COLORIMETRIC METHOD FOR THE DETERMINATION OF PENICILLIN

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A colorimetric method for the determination of penicillin has been designed. This method is based upon the following reaction.



In the above scheme, R' represents a chromogenic group.

The structural formula for the penicillins employed above is the  $\beta$ -lactam constitution suggested by the Committee on Medical Research and the Medical Research Council (1). The alternative azlactone structure would lead to the same constitution for the addition product II, which can be separated from the reactants by virtue of its solubility in aqueous alkali and its extractability from weakly acidic solution by an organic liquid.

A number of amines were evaluated in an effort to select the most suitable reagent for the test. In the course of this search an azo dye, N-(1-naphthyl-4-azobenzene)-ethylenediamine, was found to possess suitable physical and chemical properties. In the test procedure, it gives a 90 per cent yield of the amide when the condensation reaction is permitted to proceed over an 18 hour interval. The excess reagent is readily separated from the reaction medium. The amide, being colored, can be measured with good precision at high dilution and without further manipulation.

During the development of the test, methodological variations were introduced from time to time. The procedure ultimately adopted involves the following considerations. The reagent I is a strong base, and the primary amine is the stronger of the two basic groups. In the presence of acetic acid, which accelerates the reaction, the reagent condenses with the free acid of penicillins in a water-immiscible solvent. Extraction of the

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organic solution with sodium hydroxide removes the condensation product as the sodium salt, leaving the excess reagent in the organic phase. The weakly basic condensation product II is then extracted from acidic solution. Any of the strongly basic reagent I that may have passed into alkaline solution remains in the acidic, aqueous phase. Details of the method are set forth in the experimental part.

### EXPERIMENTAL

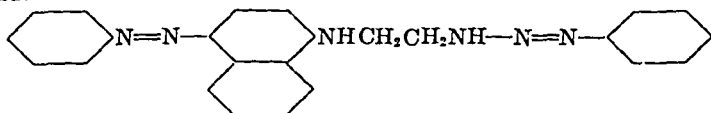
*Preparation of Dye*—To 12.5 gm. of aniline hydrochloride in 625 cc. of water, containing 16 cc. of concentrated hydrochloric acid, at 0–5°, are added dropwise, with stirring, 125 cc. of water containing 8.0 gm. of sodium nitrite. After 15 minutes at 0–5°, the excess of nitrite (starch-KI) is decomposed with solid ammonium sulfamate.

To a solution of 25 gm. of *N*-(1-naphthyl)ethylenediamine dihydrochloride (2) in 1250 cc. of cold water containing 40 cc. of concentrated hydrochloric acid the cold diazonium salt is added with stirring, at room temperature. The mixture is allowed to stand at room temperature for 10 to 15 minutes and warmed to 50–60°. After 30 minutes at this temperature, the mixture is chilled and strong sodium hydroxide is added until the color changes from a deep purple to an orange. The pH of the solution must be above 9.6. The product is filtered and dried.

The crude yield at this point is about 29 gm., but this represents a mixture. This can be separated by the use of Skellysolve C (b.p. 85–100°). The dry solid is powdered and refluxed briefly in 2500 cc. of the solvent, and an insoluble residue is removed.<sup>1</sup> When cold, the filtrate yields a crystalline product, m.p. 105–107°. The yield is about 70 per cent.<sup>2</sup> For use in the test, it is recrystallized from Skellysolve (10 gm. per liter). From this solvent, the product is obtained, with very little loss, as thick, red crystals, m.p. 107–108°. When recrystallized from 50 per cent aqueous methanol, it is obtained as orange-colored needles, m.p. 103–103.5°. Both forms are interchangeable.

$C_{18}H_{18}N_4$ .	Calculated.	C 74.45, H 6.25, N 19.29
	Found.	" 74.65, " 6.35, " 19.58

<sup>1</sup> A fraction of this insoluble residue was obtained as red crystals, m.p. 128–130°. This product does not possess a primary amine group, and appears to be a diazoamino compound.



$C_{24}H_{22}N_6$ .	Calculated.	C 73.10, H 5.58, N 21.32
	Found.	" 72.82, " 5.55, " 21.75

<sup>2</sup> No attempt was made to develop the method for preparing this dye.

On the addition of less than a molecule of dry hydrogen chloride to an ethereal solution of the basic dye, golden, needle-like crystals are formed. An excess of hydrogen chloride yields a purple crystalline product. These products, which resist purification, represent the mono- and dihydrochlorides, respectively.

**Spectrophotometry**—Aqueous solutions of the pure dye were examined in the Beckman spectrophotometer over the range 300 to 600  $m\mu$  with the results shown in Fig. 1. In 0.1 N hydrochloric acid (Curve 1), the dye yields

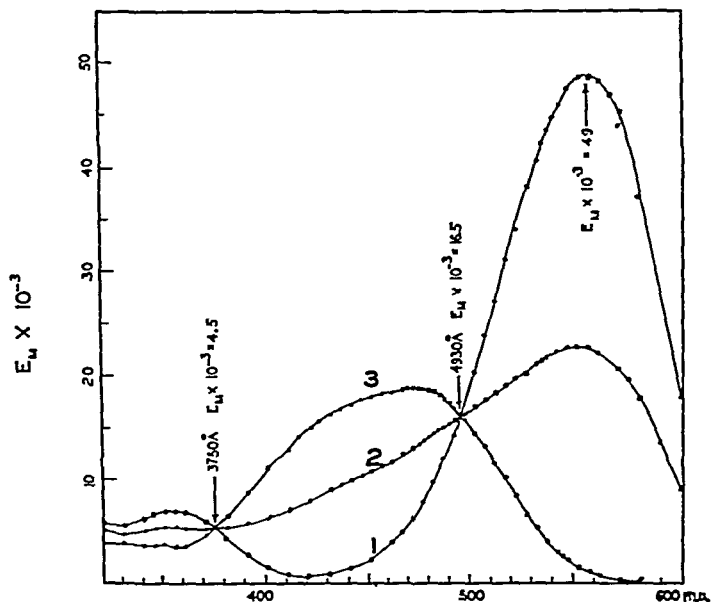


FIG. 1. The absorption of aqueous solutions of N-(1-naphthyl-4-azobenzene)-ethylenediamine in 0.1 N hydrochloric acid (Curve 1); at pH 3.0 (Curve 2); and at pH 6.0 (Curve 3).

a bluish red solution which exhibits an intense band at 555  $m\mu$  ( $E_M = 49,000$ ). The color of the solution changes to orange at pH 3.0 (Curve 2) and to yellow at pH 6.0 (Curve 3). The curves pass through isobestic points at 375 and 493  $m\mu$ . The solubility characteristics and the changes in the absorption with pH suggest that the dye is a fairly strong base, that the primary amine is the stronger of the two basic groups, and that the dye undergoes a tautomerism analogous to that exhibited by *p*-aminoazobenzene.



*Preparation of Penicillin Solutions*—Crystalline samples of penicillin G sodium salt may be weighed with the usual precautions, but commercial samples should be finely divided and should be rapidly weighed in a dry atmosphere.

All solutions are submerged in an ice bath and *must* be kept cold (0–5°) throughout this procedure. The penicillin is extracted from acidic solution with chloroform as follows:

To 5 cc. of a glycine buffer<sup>3</sup> at pH 2.0 are added 25 cc. of reagent grade chloroform and 5 cc. of an aqueous solution containing 20 to 120  $\gamma$  of penicillin. Immediately upon addition of the penicillin to the cold solutions, the system is vigorously shaken for 2 minutes. The phases are allowed to separate during 2 to 4 minutes, and the chloroform run into a chilled 25 cc. glass-stoppered graduate. About 3 gm. of anhydrous sodium sulfate are added, and 20 cc. of the superficially dried extract are added to the condensation medium as described in the test procedure.

In this process the penicillin is largely extracted from the acidic solution by chloroform, but that which remains in the aqueous phase continually undergoes spontaneous decomposition. Since equilibration cannot be effected, the temperature is kept low and the extraction time is limited to an optimum of 2 minutes.

It should be emphasized that no method involving the extraction of penicillins can give reproducible results unless the extraction process is reproducible. With the present method, end-results are probably influenced more by variations in the penicillin extraction than by any other factor. To keep these variations minimum, it is necessary to keep the temperature low, to limit the extraction time, and above all to maintain the extraction efficiency constant by uniform vigorous shaking.

*Test Procedure*—To 15 cc. of benzene containing 10 mg. of the dye there are added 20 cc. of the chloroform extract and, immediately thereafter, 5 cc. of a solution of 5 cc. of glacial acetic acid in a liter of benzene. The reaction mixture is allowed to stand at room temperature in a closed container for 3 hours ( $\pm 3$  minutes). It is then shaken vigorously for 10 seconds with 25 cc. of 0.05  $N$  sodium hydroxide in a 125 cc. separatory funnel. When both phases have separated and are crystal clear, the lower layer is slowly drawn off, 0.2 to 0.5 cc. of the organic solvent being left in the funnel. (During this phase separation disturbance of interfacial material must be avoided.) Then 25 cc. of chloroform are added to the sodium hydroxide and the whole is shaken for 10 seconds. After complete phase separation the chloroform is removed as before, 0.2 to 0.3 cc.<sup>4</sup> being left in order to

<sup>3</sup> This buffer was prepared from 600 cc. of 0.2  $M$  glycine in 0.2  $M$  sodium chloride by the addition of 0.2  $M$  hydrochloric acid (540 cc.) to a pH of 2.0.

<sup>4</sup> It may be desirable to mark funnels at the 0.3 cc. level for this purpose.

avoid loss of interfacial material. After the addition of 1 cc. of concentrated hydrochloric acid followed by 15 cc. of a mixture of 1 volume of butanol and 4 volumes of benzene, the system is shaken for 10 seconds, to transfer the red condensation product to the butanol-benzene phase. To 10 cc. of the butanol-benzene extract are added 2 cc. of a mixture of 5 volumes of concentrated hydrochloric acid and 95 volumes of absolute ethanol. (The strong acid is required to bring forth maximum tautomerism.) The color intensity is measured in a photoelectric colorimeter equipped with a No. 540 filter.

As the colored reagent distributes itself between benzene (or chloroform) and 0.05 *N* sodium hydroxide in a ratio of about 1000:1, it is obvious that the above procedure will suffice to remove essentially all of the excess dye. In this process, again, the distributions are not brought to equilibrium, because the attainment of equilibrium would require excessive shaking. The extraction procedures are consequently limited in time in order to give reproducible results. 10 seconds of shaking suffice to convert all of the condensation product to the sodium salt. The product is thus removed from the organic phase.<sup>5</sup> This time restriction does not, however, permit complete saturation of the aqueous phase with the excess dye. Conversely, in the initial 10 second chloroform wash, extraction of the free dye which dissolved in the aqueous phase is not complete. Only the final extractions are effective in this respect.

The use of concentrated hydrochloric acid is necessary in conjunction with the butanol extraction. If less acid is used, the dye is extracted as a mixture of the mono- and dihydrochlorides rather than as the dihydrochloride, and this leads to low colorimetric values (*e.g.*, see Curve 2 of Fig. 1).

Care must be exercised in removing the excess reagent in the course of the test. Because of its high tinctorial intensity traces retained by separatory funnel stop-cocks and stoppers can give rise to erroneous results. It should also be noted that the penicillin-dye amide at higher concentrations does not appear to form a true solution in 0.05 *N* sodium hydroxide, but appears to associate and agglomerate at interfaces. In order to avoid losses of product, the procedure is designed to permit cutting of the two phases on the safe side of the interface without changing separatory funnels.

Moderate variations of time and temperature in the test procedure give rise to no significant variations in the analytical results. However, there is one factor, *viz.* the agglomeration of condensation product at interfaces, which gave rise to variations in an earlier procedure. Loss of this interfacial material, which caused negative errors, has been greatly reduced in

<sup>5</sup> This salt has a real solubility in organic solvents, but the difference between the solubility of the free dye and of the sodium salt of the condensation product is sufficient to permit a satisfactory separation.

the procedure described here. Methanol (5 cc.) may be added to the aqueous alkali, and by thus dissolving *all* of the condensation product the possibility of losing interfacial solids can be eliminated, but this is not necessary. Losses, which can only occur in the first two steps of the test procedure, are avoided simply by awaiting a satisfactory phase separation

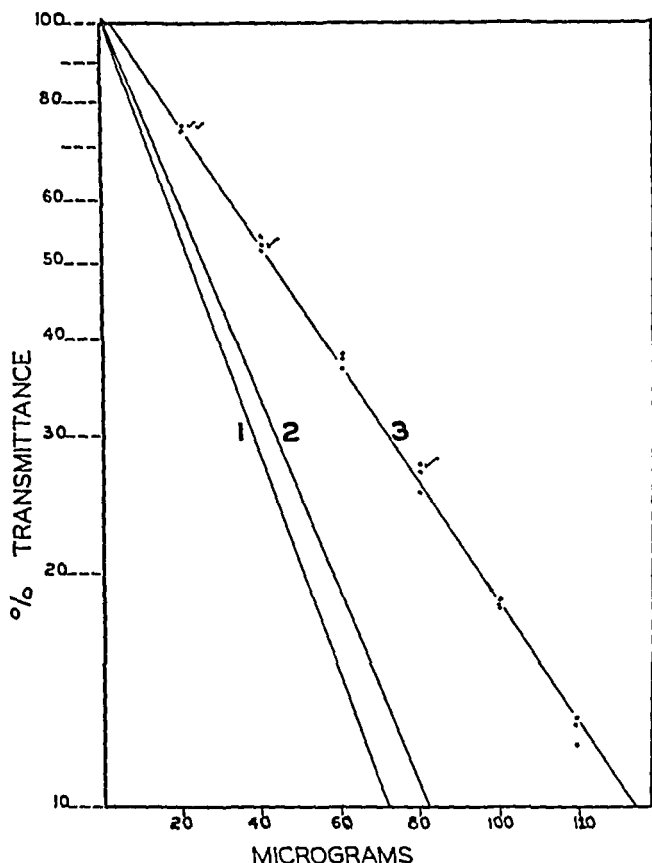


FIG. 2. Calibration data. Transmittance of pure solutions of the dye (Curve 1). Data recalculated in terms of the sodium salt of penicillin G (Curve 2). Calibration data (Curve 3).

and then by removing the organic phase carefully. We believe that with reasonable care significant errors will not occur in the performance of the test procedure.

*Calibration*—Standard solutions of the reagent, prepared in acidified butanol-ethanol, as in the procedure, were measured in the photoelectric colorimeter equipped with filter No. 540. Three such sets of solutions pre-

pared with different samples gave data (Fig. 2, Curve 1) in good agreement with Beer's law, and the agreement between samples was good. Assuming that solutions which contain the specific chromogenic grouping are directly comparable at 540  $m\mu$ , and that the absorption is inversely proportional to the molecular weight, it is possible, stoichiometrically, to express these data in terms of the sodium salt of penicillin G (Fig. 2, Curve 2). Comparison

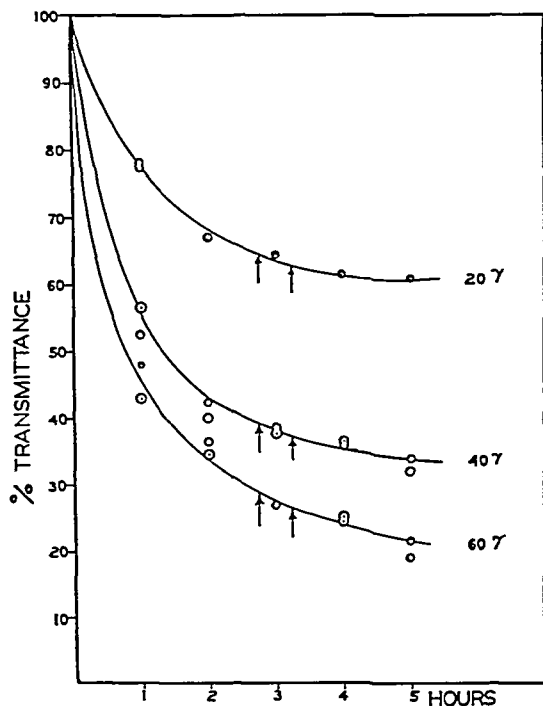


FIG. 3. Rate of condensation of penicillin and dye under test conditions

of these recalculated data with calibration data obtained, following an 18 hour period of condensation between penicillin and the colored amine, indicated a yield of approximately 90 per cent. The rate of condensation, however, was excessively slow. It was then found that acetic acid markedly accelerates the rate of condensation. The rate was increased with increasing concentrations of acetic acid up to 0.0185 gm. per reaction tube, but beyond this concentration and up to 0.0554 gm. of acetic acid per reaction tube the rate was not augmented. Since in these experiments the

benzene solution contained 9 to 27 moles of acetic acid per mole of amine, it may be concluded that penicillin condenses more rapidly with the acetate of the free primary amine than with the free base.

A number of test solutions containing different concentrations of penicillin were extracted with 0.05 N sodium hydroxide at hourly intervals. The data are presented in Fig. 3. These show that the condensation is largely effected within the first 3 hours. The yield is about 70 per cent. The curves indicate that a  $\pm 15$  minute variation in the condensation time introduces an analytical variation of only  $\pm 2$  to 3 per cent. This variation is reduced to negligible proportions by restricting the condensation period to 3 hours,  $\pm 3$  minutes. For some purposes, a 2 hour interval would be desirable. It may be possible to standardize on a 2 hour basis with but little loss of sensitivity and reproducibility.

In a series of 66 blank determinations four different operators obtained an average value of the transmittance,  $T$ ,  $= 96.6 \pm 0.54$  per cent. Of these readings, forty-five agreed with the average within 0.0 to 0.5, thirteen agreed within 0.5 to 1.0, and eight agreed within 1.0 to 1.6 per cent. Lots of chloroform or benzene which give lower blanks should not be used in this test.

In four separate trials, analysis of a series of six standard solutions of pure penicillin yielded the data shown in Fig. 2 (Curve 3). The average deviation of these data from the best straight line, expressed in per cent analytical error, is  $\pm 1.5$  per cent. From a long series of such trials it was concluded that in the hands of a well trained and careful operator the present method may be expected to involve an average error of about 1.5 per cent, whereas in the hands of young and relatively untrained operators an average error of 3 to 5 per cent may be anticipated.

It should be noted that the calibration curve (Curve 3, Fig. 2) does not pass through 100 per cent transmittance at zero concentration. The divergence increases with diminishing penicillin concentration and is apparently a function of dilution. It is reasonable to assume that this divergence is produced either by inactivation of part of the penicillin or by loss of part of the condensation product in the extraction procedures.

*Sensitivity*—The method, as presented, was designed primarily for application to the assay of commercial samples of penicillins. For this purpose, sensitivity is not critical, 10 to 100  $\gamma$  of active material being readily available. With culture media, broths, urine, etc., for which the method is equally useful, a minimum of 10  $\gamma$  is required, but by simple reduction of volumes it is possible to estimate quantities as small as 1  $\gamma$ .

*Specificity*—This method possesses certain specificity requirements. For a substance to be measured as a penicillin, it must be water-soluble at pH 7.0; it must be extractable by chloroform from aqueous solutions at pH

2.0; it must condense completely with the reagent within a limited period of time; the product must possess a carboxyl group; and finally, the condensation product must not possess strongly basic properties. That some potential interfering substances do not meet this final requirement is evidenced by the fact that variable amounts of colored materials are left in the acid phase in the course of analysis of commercial samples of penicillin. In this connection, it may be noted that a variety of degradation products of penicillin has been tested with no significant evidence of interference.

To obtain confirmatory evidence that the products which give the color test are indeed penicillins, a number of experiments were performed with crystalline penicillin G which had been inactivated by various procedures. (1) 100  $\gamma$  of crystalline penicillin, and equivalent amounts of commercial samples, were maintained in 5 cc. of a sodium acetate-hydrochloric acid buffer at pH 2.3 for 2 hours. (2) 200  $\gamma$  quantities were maintained in 1 cc. of 0.05 N sodium hydroxide for 2 hours. (3) 100  $\gamma$  quantities were maintained in 3 cc. of solutions containing 5 mg. of thiolethylamine at pH 7.2 for 1 hour. (In Experiment 3a, Sample B was treated instead with 0.5 per cent cysteine at pH 7.3 for 2 and 3 hour periods.) (4) 200  $\gamma$  quantities were maintained at 37° for 1.5 hours at pH 6.72 in the presence of 5 cc. of a penicillinase-containing broth.<sup>6</sup> The culture was centrifuged to remove the cells, and the penicillinase-rich supernatant was used directly. Samples of the broth were autoclaved at 15 pounds pressure for 20 minutes. This treatment served to inactivate the enzyme. Control experiments with the autoclaved preparation produced no significant destruction of penicillin.

From the data recorded in Table I it may be inferred that the products of these methods of inactivation do not react with the colored amine in the test procedure. In all cases, the decrement observed with the commercial samples of penicillin was identical, within experimental error, with the decrement observed with crystalline penicillin. Hence, it may be concluded that the products present in commercial penicillin preparations which give the color test have the stability characteristics of active penicillins.

Despite these specificity requirements, interference by other types of substances may be anticipated; for example, ether-soluble keto acids may react with the colored amine to give alkali-soluble products of the Schiff base or aldehyde-ammonia type, and lactones, azlactones, and esters may react to give amidic compounds. Nevertheless, when applied to a large number of commercial samples of penicillin, the method gave results which were satisfactory in comparison with microbiological data (correlation coefficient = +0.964). Further, comparison of the stability characteristics of crystalline penicillin G and of the active principle of commercial

<sup>6</sup> This broth was obtained after 47 hours growth of Foster's Culture 164 on yeast extract.

preparations also indicated that penicillin was measured specifically. When applied to low potency samples, and to broths, however, interfering substances may be detected. If significant amounts are present (which is seldom the case), these can be eliminated readily as described below.

*Interfering Substances*—The marked lability of the penicillin affords a ready means of differentiating the active principle from inactive interfering substances. A given sample may be analyzed as usual to give a "total" concentration. An aliquot, exposed to the action of some inactivating agent, may be analyzed to give a "non-penicillin" value. The "total" minus the "non-penicillin" value would be expected to give a close approximation of the true penicillin concentration.

TABLE I  
*Inactivation of Crystalline and Commercial Penicillin Preparations As Measured Colorimetrically*

Experiment No.	Treatment	Per cent decomposition				
		Crystalline penicillin G	Commercial samples			
			A	B	C	D
1	2 hrs. at pH 2.3	69	70	65	68	68
		70	70	65	70	69
2	2 " in 0.05 N NaOH	91	85		84	89
		92	87		85	
3	Thiolethylamine, pH 7.2	67	69	63	56	67
3a	Cysteine, pH 7.3	81		80		
		81		78		
4	Penicillinase, pH 6.7	89	83	84	81	87
		90	84	85	81	87

The recent preparation of potent samples of penicillinase suggests that the "non-penicillin" fraction may best be determined following penicillinase inactivation of the active principle. At the time this work was performed, potent penicillinase preparations were not available. Consequently, alkaline inactivation was studied, and aliquots were routinely exposed to the action of 0.2 N sodium hydroxide at room temperature for 1 to 2 hours to give the "non-penicillin" value which was subtracted from the "total." This manipulation of the data was predicated on the assumptions (a) that the penicillin in a given sample is completely destroyed by the alkali treatment, and (b) that this treatment neither generates nor destroys the interfering substances in the sample. These assumptions have been justified experimentally. Crystalline samples of penicillin G were completely inactivated (*i.e.*, gave negative color tests) when treated with alkali in the presence or absence of commercial penicillin or broths. Commercial

preparations and broth samples which possessed no antibiotic activity but contained measurable amounts of interfering substances gave identical analytical results before and after alkali treatment. Further, active penicillin samples which contained significant amounts of interfering substances gave identical results both after 1 and 2 hours of exposure to alkali. The "non-penicillin" value may therefore logically be subtracted from the "total" value.

Regardless of the means selected to inactivate the penicillin, it should be noted that the colorimetric calibration curve is characteristic for penicillin and may be used directly for the determination of the "total" penicillin content of materials, but it is not characteristic for the "non-penicillin" fraction which is composed of unknown and variable materials. Consequently, it is not permissible to read the "non-penicillin" value directly from this calibration curve. Direct use of this curve for the estimation of the "non-penicillin" value would lead to the obvious error of assigning a value of 3.7  $\gamma$  to a solution which exhibits 100 per cent transmittance. In order to correct this error, it is necessary merely to subtract the value indicated by the calibration curve at 100 per cent transmittance from the apparent "non-penicillin" value.

*Application to Broth*—The colorimetric method was used in the analysis of penicillin broth samples as follows: Samples of broth, selected at random, were treated with a filter aid (0.5 gm. of Hyflo Super-Cel was added to 50 cc. of broth) and the mixture was shaken. The broth was filtered through a Büchner filter bed which was precoated with a thin layer of Super-Cel. Aliquots of the filtrate (1 to 6 cc., depending on the potency of the broth) were analyzed at levels planned to contain approximately 100 and 200  $\gamma$  of penicillin. This gave the "total" value in duplicate. Two equal aliquots were treated with 0.4 N sodium hydroxide to give solutions 0.2 N in alkali. These aliquots were permitted to stand at room temperature for 1 to 2 hours to inactivate the penicillin. The solutions were then adjusted to volume and the analyses were completed as usual.

In spite of the satisfactory recovery of added penicillin G ( $98.7 \pm 4.0$  per cent, maximum deviation, 13 per cent) obtained in the chemical analysis of a large number of broths, the chemical data were consistently lower than those obtained by means of microbiological methods. This discrepancy suggests that either the microbiological method is not entirely specific, or the broth samples contained a mixture of penicillins of high biological potency.

#### DISCUSSION

Some comment on the existence of different varieties of penicillin seems appropriate. It is now generally recognized that not only does the antibiotic potency of a single variety of penicillin differ with different test or-



ganisms, but the different penicillins evoke different responses with the same organism. It is therefore evident that a direct correlation of chemical and microbiological assay values cannot be expected when mixtures of the various penicillins are analyzed. Chemical and microbiological values will approximate each other only as the concentration of one kind of penicillin increases, or as the activities of the different kinds approach each other. Chemical procedures, like that here described, which are based upon a key functional group common to all penicillins, yield molar values for all varieties of penicillin without differentiation between the varieties.

#### SUMMARY

A colorimetric method for the determination of total penicillins has been designed for the analysis of commercial preparations and of broth. The method involves the interaction of an intensely colored primary amine, N-(1-naphthyl-4-azobenzene)-ethylenediamine, with the penicillins to yield amidic products containing acidic groups. The preparation of this reagent is described. Measurement of the total penicillins is made in terms of the dye bound to the antibiotic. The method is comparatively simple, being carried out almost entirely in a single separatory funnel. A capable analyst can, by means of this method, carry out large series of analyses at one time.

The colorimetric method appears to be more precise and reproducible than existing microbiological methods of assay. Its specificity has been considered in some detail, and the elimination of potential interfering substances by means of a differential method of analysis has been discussed. The difficulty in assigning a biological activity, from chemical analysis, to a solution which contains a mixture of penicillins has been considered briefly.

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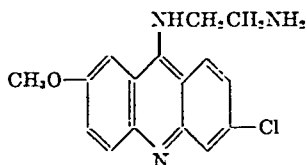
# A RAPID MICROMETHOD FOR THE FLUOROMETRIC DETERMINATION OF PENICILLIN

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The colorimetric method for the determination of penicillin, described in the preceding report (1), is more precise than existing biological methods but is limited in sensitivity. In order to extend the range of applicability of the method by increasing its sensitivity, the strongly fluorescent compound 2-methoxy-6-chloro-9-( $\beta$ -aminoethyl)aminoacridine



has now been used in place of the aminoazo compound originally employed. Quantitative measurements of small amounts of the penicillins have thus been achieved. As the aminoacridine condenses with the penicillins at a more rapid rate than does the azo dye, it has been possible to reduce the condensation time to 1 hour. Details of the method are set forth in the experimental part.

## EXPERIMENTAL

*Synthesis of Aminoacridine*—5 gm. of halocrin (6,9-dichloro-2-methoxyacridine) were warmed for 20 minutes on a steam bath with 100 cc. of anhydrous ethylenediamine. The solid dissolved within 10 to 15 minutes. The reaction mixture was then boiled for 3 minutes and cooled to room temperature. About 60 cc. of the excess ethylenediamine were recovered by vacuum distillation. To the cooled residue, 250 cc. of cold water were added; the precipitate was dissolved in 500 cc. of cold 0.1 N hydrochloric acid and the solution was filtered. The filtrate was treated in the cold with 30 per cent sodium hydroxide to maximum precipitation. The thoroughly dried product (5.4 gm.) was recrystallized from 3.0 liters of Skellysolve C. The yield of yellow crystals, m.p. 141–142°, was 4.5 gm., or about 80 per cent. About 0.9 gm. more of the pure product was secured by concentra-

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ting the mother liquor. After recrystallization from the same solvent, the compound melted at 142–143°.

$C_{16}H_{16}N_3OCl$ . Calculated. C 63.68, H 5.34, N 13.93  
Found. " 63.90, " 5.26, " 14.04

*Spectrophotometry*—With the Beckman spectrophotometer, aqueous solutions of the aminoacridine were examined in the near ultraviolet and visible regions of the spectrum with the results shown in Fig. 1. In acidic

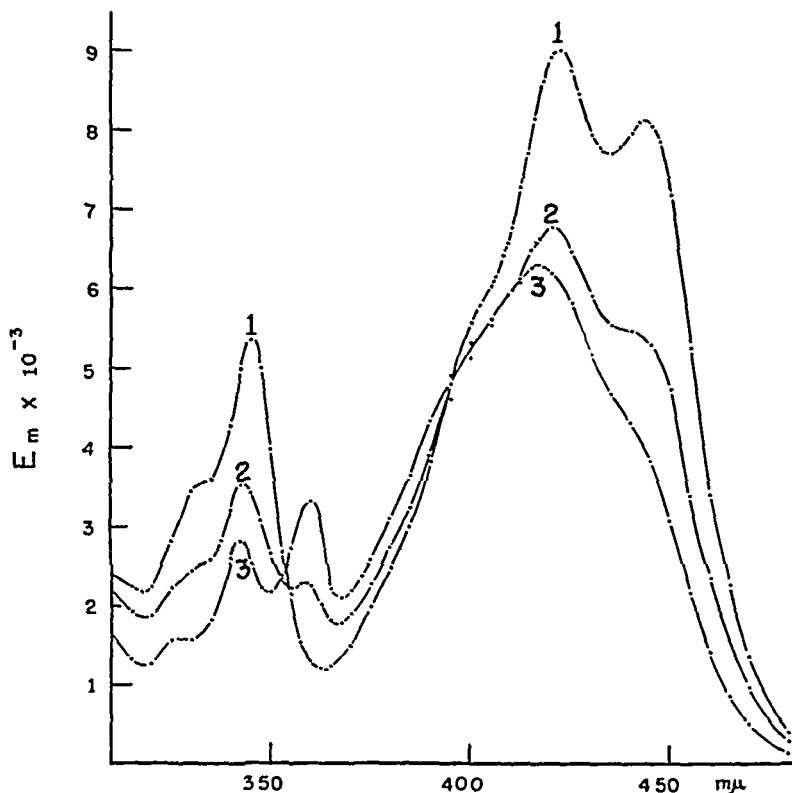


FIG. 1. The absorption of aqueous solutions of 2-methoxy-6-chloro-9-( $\beta$ -aminoethyl)minoacridine at pH 1.4 (Curve 1), at pH 8.1 (Curve 2), and at pH 9.8 (Curve 3).

solution (pH 1.4) the product exhibited a band at 345  $m\mu$  and a broad band in the visible region with maxima at 421 and at 443  $m\mu$ . The millimolecular extinction coefficients at these wave-lengths were 5.35, 9.00, and 8.10, respectively. The absorption of the solution changed with increasing pH. The change was pronounced at pH 8.1 and essentially complete at pH 9.8. At the latter pH, there were small bands at 343 and 360 and a

broad band with a maximum at 417  $\mu$ . The millimolecular extinction coefficients at these wave-lengths were 2.82, 3.32, and 6.30, respectively. These data indicate that the product is a homologue of atabrine.<sup>1</sup> One may infer that the product is an even stronger base than atabrine, and that the primary amino group is the strongest of the three basic groups. Iso-bestic points were observed at 353 and 395  $\mu$ . One may therefore infer, further, that the product exhibits a tautomerism, which is similar to that observed with atabrine (2) and which is limited in the pH range under consideration to two molecular species.

*Preparation of Penicillin Solutions*—All solutions are submerged in an ice bath and must be kept cold (0–5°) throughout. The procedure is carried out as follows:

To 8 cc. of an aqueous solution containing 0.0625 to 0.625  $\gamma$  of penicillin per cc., there are successively added 12 cc. of reagent chloroform and 2 cc. of a Sørensen glycine buffer at pH 2.0. The mixture is immediately shaken vigorously for 30 seconds. The phases are allowed to separate during 30 to 60 seconds. The chloroform layer is run into a chilled, glass-stoppered graduate and rapidly dried with 1 to 2 gm. of anhydrous sodium sulfate.

Application of the extraction method to larger volumes of more dilute solutions may be accomplished in three ways. A double extraction of the penicillin may be employed, the penicillin may be extracted with proportionally smaller volumes of chloroform, or the chloroform extract may be concentrated prior to condensation. These procedures were studied as follows:

*Double Extraction*—80 cc. of each of three solutions (containing 0.00625 to 0.025  $\gamma$  of penicillin per cc.) were extracted as described, but all volumes were increased 10-fold, and 40 cc. of each of three solutions (containing 0.0125 to 0.05  $\gamma$  of penicillin per cc.) were similarly extracted, all volumes being increased 5-fold. The chloroform extracts (volumes being 120 and 60 cc., respectively) were shaken for 30 seconds at 0–5° with 8 cc. of a Sørensen phosphate buffer at pH 7.0. The chloroform layer was discarded and 12 cc. of fresh chloroform and 2 cc. of 0.4  $N$  hydrochloric acid were added. The penicillin was thus reextracted and the analyses were completed as usual with satisfactory results.

*Reduced Chloroform Volume*—The above experiments were repeated and the volumes were multiplied by the appropriate factor of 5 or 10, but only 12 cc. of chloroform were used for each extraction. The chloroform extract was then used directly in the test procedure and the analyses were completed as usual. Satisfactory data were obtained in analyses of the solutions containing 0.0125 to 0.05  $\gamma$  of penicillin per cc., but excessive er-

<sup>1</sup> This compound has presumably been synthesized heretofore, but a description of the compound and its synthesis has not been found.

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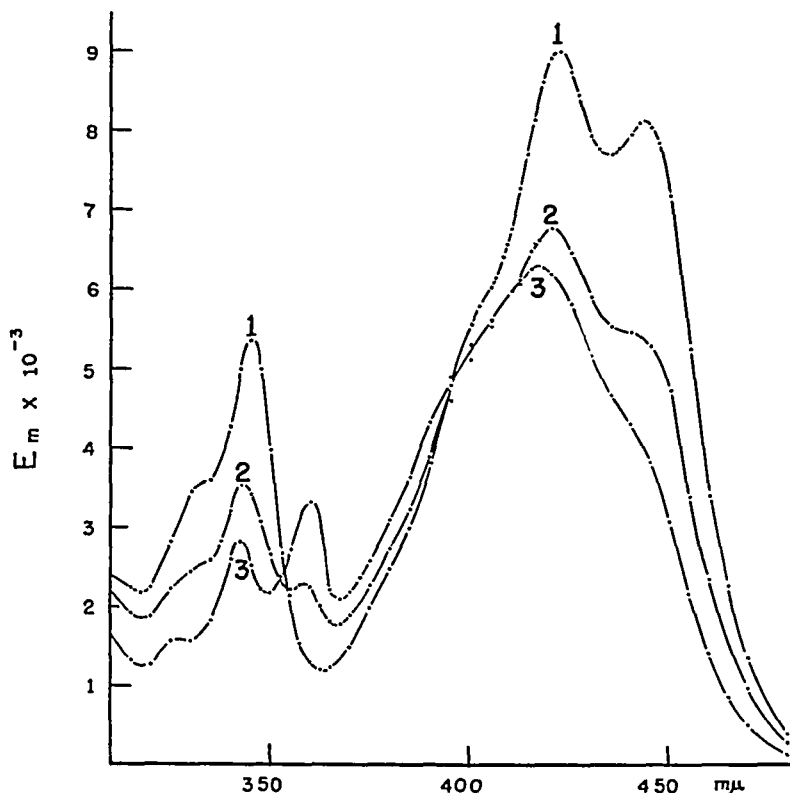


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rors occurred in the analysis of the more dilute series of solutions. The analytical results are lowered by distribution effects, and a corresponding calibration curve is required if this procedure is used.

*Concentration of Chloroform*—With proportionally larger volumes throughout, dilute penicillin solutions were extracted and the *thoroughly* dried chloroform extracts were evaporated to an appropriate volume in a current of dry air at 0–5°. Analyses gave satisfactory results.

Of the three methods of concentration, the first appears to be the one of choice, although the second is the simplest. The third method is not suited to routine use.

In the application of this process to urine, the filtered sample is diluted 1:50 or more, and 8 cc. of the diluted urine are extracted as usual. This dilution limits the sensitivity of the method somewhat, but the limitation is not significant in studies of the urinary excretion of therapeutic doses of penicillin, nor is the dilution obligatory. Smaller dilutions may be used, but fluorescent urinary pigments then become measurable and larger blanks must, consequently, be subtracted from final readings.

Application of the extraction process to whole blood or plasma involves a preliminary preparation of a protein-free filtrate. By the Haden modification (3) of the Folin-Wu method, 8 cc. of centrifugate are readily obtained, and the pH is adjusted to pH 2.0 by the addition of 2 cc. of 0.1 N hydrochloric acid instead of the glycine buffer recommended above. Apparently, some sorption<sup>2</sup> of penicillin on the blood proteins occurs during this procedure, because when penicillin was added to whole blood in concentrations of 1 and 2  $\gamma$  per cc. the average recovery in a limited series of nine determinations was 77 ( $\pm 9$ ) per cent. In order to correct for this discrepancy, final answers are divided by the factor 0.77. The 1:10 dilution of blood limits the sensitivity of the method. Analyses may be completed upon 1 cc. of whole blood or plasma containing 0.625  $\gamma$  of penicillin per cc. or more, but in order to measure concentrations as low as 0.125  $\gamma$  per cc. 5 cc. of blood are required.

It should be emphasized that no method involving the extraction of penicillin can give reproducible results unless the extraction process is reproducible. In order to keep variations at a minimum, it is necessary to keep the temperature low, to limit the extraction time, and to maintain the extraction efficiency constant by uniform vigorous shaking. The aqueous phase must have pH 2, but the penicillin which is unstable must not be permitted to remain in this phase for significant periods of time. Glassware must be scrupulously clean and must not contain traces of fluorescent stop-cock grease, and solvents must be free of fluorescent contaminants.

<sup>2</sup> Sorption effects appear to be diminished by precipitating the blood proteins at higher dilutions. Direct extraction from diluted whole blood was not studied.

*Test Procedure*—To 5 cc. of benzene containing 10 mg. of the aminoacridine, there are successively added 2 cc. of reagent grade acetone, 10 cc. of the chloroform penicillin extract, and 5 cc. of a solution containing 10 cc. of glacial acetic acid in a liter of benzene. The reaction mixture is allowed to stand in a closed container at room temperature in the absence of light for 1 hour,  $\pm 5$  minutes.<sup>3</sup> The reaction mixture is then vigorously shaken for 10 seconds in a 125 cc. separatory funnel with 10 cc. of 0.5 N sodium hydroxide. After phase separation, the lower, organic layer is removed. The alkaline solution is shaken vigorously for 5 seconds with two successive 5 cc. portions of chloroform, the chloroform layer being removed after each phase separation. The alkaline solution is acidified with 1 cc. of glacial acetic acid and the condensation product is extracted from the reaction mixture with 15 cc. of butanol-benzene solution (1:2 by volume) by shaking vigorously for 30 seconds. The aqueous layer is discarded and the organic layer shaken for 30 seconds with 10 cc. of 5 per cent aqueous acetic acid. The aqueous layer is discarded and 50 cc. of chloroform followed by 15 cc. of 0.5 N sodium hydroxide are added. The condensation product is transferred to the aqueous phase by shaking for 30 seconds. The lower organic layer is discarded and 1 cc. of concentrated hydrochloric acid is added to the alkaline solution. The acidic solution is placed in the cuvette and its fluorescence intensity measured.

*Fluorescence Measurements*—As the aminoacridine used in the test procedure is a close analogue of atabrine, it may be expected to possess similar fluorescence characteristics. This assumption is in agreement with the results of Butler's studies (4) of the distribution of radiant energy in the fluorescent spectra of atabrine and other acridine derivatives including metabolic products of atabrine.<sup>4</sup> On this assumption, measurements of the aminoacridine and of the condensation product were made in the Pfaltz and Bauer fluorophotometer, model B, equipped with the filters recommended (5, 6) for the measurement of atabrine. A No. 5113 Corning glass filter, 2 mm. in thickness, was placed in the path of the incident beam, and a No. 3385 Corning glass filter, 2 mm. in thickness, was placed between the sample and the photocell. Equally good results were obtained with the Pfaltz and Bauer instrument equipped with the riboflavin filters sold by these manufacturers.

Before a series of measurements is made, the fluorophotometer must be adjusted to a constant light intensity. This may be done conveniently by means of an appropriate fluorescent glass block (7), but in the present work

<sup>3</sup> At the end of this time the extractions are begun and these should be completed as rapidly as possible with a minimum exposure to light. A shaded hood is suitable for this purpose. Low actinic Corning glassware may also be suitable.

<sup>4</sup> Personal communication from Dr. Butler.



this was done with a solution of 0.8  $\gamma$  of the aminoacridine in 16 cc. of 4 N hydrochloric acid.<sup>5</sup> The fluorescence of this standard solution was arbitrarily adjusted to 60 per cent of the galvanometer scale. To eliminate the influence of variations in the electric current, two equally balanced cuvettes were employed. One was used for the standard and the other for the unknown solution, and the instrumental setting was checked against the standard before and after reading unknown solutions.

It is necessary to subtract from final readings the values obtained in blank determinations. In a series of thirteen blank determinations the average value was 9.1 ( $\pm 0.9$ ). This value may be subtracted from final readings, but, since the blank fluorescence may vary with different samples, it is preferable to perform blank determinations with each series of analyses.

*Calibration*—A series of aqueous solutions containing 2 and 3  $\gamma$  of penicillin was extracted with chloroform. The penicillin so extracted was allowed to condense with the aminoacridine, but the condensation time was varied by shaking out the reaction mixtures with the 0.5 N sodium hydroxide at 10 minute intervals. The analyses were then completed as usual. The analytical data indicated that the condensation reaction reaches a maximum at 30 minutes, and that the yield of product remains constant thereafter. Variations of  $\pm 5$  minutes in the condensation time, which was arbitrarily limited to 1 hour, produce no detectable errors.

In a series of twenty-four analyses of standard solutions containing 0.0625 to 0.625  $\gamma$  of penicillin per cc., the average galvanometer deflection per microgram of penicillin was 14.1 ( $\pm 1.4$ ) per cent of the scale. Of the twenty-four readings at hand, fifteen deviated by less than 1.5, eight deviated by 1.5 to 2.5, and one deviated by 4 units from the mean of 14.1. The average deviation from the mean, expressed in terms of analytical error, is  $\pm 10$  per cent, which is about the precision to be expected for fluorometric methods.

*Sensitivity*—It is frequently possible to increase the sensitivity of fluorescence methods by adding to the test solution substances which enhance its fluorescence. For this reason substances known to augment the fluorescence of aqueous atabrine solutions were investigated. The fluorescence intensity of the aminoacridine was maximum in approximately 4 N hydrochloric acid. Sulfuric acid was essentially equivalent to hydrochloric acid, whereas acetic acid gave much less satisfactory results. The Ferrari reagent (6) gave no appreciable enhancement.

The method, as presented, is directly applicable to solutions containing 0.0625 to 0.625  $\gamma$  of penicillin G per cc. For more dilute solutions, larger volumes may be concentrated as described above. The sensitivity of the

<sup>5</sup> Such solutions are stable for at least 1 week. Higher concentrations in 0.01 N acid are stable for longer periods of time.

method, expressed in terms of the minimum weight of penicillin rather than its concentration, is limited by the volume of solution required for the fluorometric measurement. With a fluorophotometer capable of recording the fluorescence of 1.5 cc. of solution, instead of the 15 cc. of solution required for the Pfaltz and Bauer instrument, the sensitivity can be increased by a factor of 10 by a simple reduction of volumes throughout the test procedure.

*Specificity*—All of the specificity requirements incorporated in the colorimetric method for the determination of penicillin (1) are inherent in the fluorescence method.

#### SUMMARY

A fluorometric method for the determination of small amounts of penicillin has been described. The method is precise to  $\pm 10$  per cent, and may be used by a trained operator to complete eight analyses in about 2 hours. Preliminary results indicate that the method is applicable to the analysis of blood and urine.

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# AMINO ACIDS IN THE PRODUCTION OF GRANULOCYTES IN RATS

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The importance of *Lactobacillus casei* factor (LCF, "folic acid") in the production of polymorphonuclear granulocytes and erythrocytes in rats has been demonstrated (1-3). More recently it was found that limitation of food intake (4) has a profound effect upon granulocyte production. Further, when rats are fed diets of low protein content, severe granulocytopenia and anemia develop uniformly (5). The granulocytopenia is effectively corrected only by the combined administration of LCF and casein or LCF and a mixture of purified amino acids. In the present study the identity of the amino acids required for the production of granulocytes has been determined. Some data on the amino acid requirements for erythrocyte production are also reported.

## EXPERIMENTAL

For the production of granulocytopenia, albino rats within 1 week after weaning were fed a protein-free, basal diet (No. 1055<sup>1</sup>) as in previous studies (5). Of 772 rats (716 Osborne and Mendel, 56 National Institute of Health strain) fed this diet, 636 survived 18 days or more and granulocytopenia was noted in 511 (80 per cent). These granulocytopenic rats were the animals used in the studies for the determination of the effectiveness of various proteins and amino acid mixtures in the production of granulocytes. The granulocytopenia was noted after an average of 21 days (range 14 to 43) on the basal diet. The average body weight was 44 gm. (range 31 to 65) when the rats were placed on the basal diet and 30 gm. (range 22 to 44) when granulocytopenia was first noted.

Total white blood cell counts, polymorphonuclear granulocyte counts, and hematocrit determinations were made as previously described (3).

<sup>1</sup> The casein-free diet, No. 1055, consisted of anhydrous dextrose (Merck, U. S. P.) 86.76 gm., Crisco 8.0 gm., Salt Mixture 550 (3) 4.0 gm., ferric citrate 1.16 gm., and copper sulfate·5H<sub>2</sub>O 0.08 gm. Into this diet were incorporated 1 mg. of thiamine hydrochloride, 2 mg. of riboflavin, 1 mg. of pyridoxine hydrochloride, 4 mg. of calcium pantothenate, 2 mg. of niacin, 200 mg. of choline chloride, 0.001 mg. of biotin, and 0.4 mg. of 2-methyl-1,4-naphthoquinone. Twice weekly each rat received a supplement of 0.25 cc. of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola) and once weekly 3 mg. of  $\alpha$ -tocopherol in 0.03 cc. of ethyl laurate.

Granulocytopenia was considered to be present when the polymorphonuclear granulocytes numbered 500 or fewer cells per c.mm.

The development of granulocytopenia usually preceded the development of a severe anemia. Of the 64 rats without granulocytopenia but having hematocrit values under 30 volumes per cent, twenty-five developed granulocytopenia in 2 days (average) and the remaining rats survived only 4.4 days (average). The survival of granulocytopenic, non-anemic rats without adequate treatment was equally poor. Since at least 8 to 10 days are required for evaluating erythrocyte production, these experimental conditions were inadequate for this type of study. Therefore, the major emphasis in these experiments has been placed upon the evaluation of granulocyte production. However, the routine determination of hematocrit has yielded some significant data concerning erythrocyte production.

Treatment to correct the granulocytopenia was started on the day of its detection. Administration of synthetic LCF<sup>2</sup> (100  $\gamma$  daily by mouth) for 4 days produced an increase in the average granulocyte count from 316 to 936 cells per c.mm. (Table I). In none of thirteen rats treated in the previous study (5) or in twenty-nine rats treated in the present study (Table I) did the granulocyte level exceed 1800 cells per c.mm. The administration of casein in the absence of LCF was usually followed by a progressive decline in the number of granulocytes. Therefore, in tests to determine the influence of various proteins and amino acids on the production of granulocytes, LCF has been administered routinely (100  $\gamma$  daily by mouth) along with the substance under test. The response of an individual rat to treatment has been considered "significant" in this test when the granulocyte recount after 4 days treatment exceeded 2000 cells per c.mm. The pretreatment values cited in Tables I, II, and III include only the values obtained from rats which survived the indicated treatment periods. No differences existed between the average pretreatment values obtained from rats which survived and those from rats which failed to survive the treatment period.

In testing for the effectiveness of various proteins and amino acids, the substances were incorporated into the diet by replacing equal weights of dextrose. The proteins were administered at an 18 per cent level, except in the case of casein with which 30 and 50 per cent levels were also tried. When amino acids were used to supplement deficient proteins, the combined weight of the natural amino acids and protein made up 18 per cent of the diet. In all cases in which racemic acids were used, only the natural form contained in them was considered.<sup>3</sup> Mixtures of amino acids were admin-

<sup>2</sup> The synthetic LCF (6) was furnished through the courtesy of Dr. E. L. R. Stokstad and Dr. B. L. Hutchings of the Lederle Laboratories, Inc.

<sup>3</sup> Throughout this study, the amino acid isomers used were as indicated in the foot-

istered in the combinations and weights indicated in Table II. The proteins used were casein (Labco), powdered egg white (Armour), zein (Corn Products Refining Company), gelatin (U. S. P., Baker), oxidized casein (prepared according to Toennies (7)), and dried human plasma.<sup>4</sup>

During the period of test, observations were made of food intake and body weight.

*Effects of Various Proteins on Granulocyte Production*—The data in Table I indicate that, while LCF alone or casein alone was ineffective in correcting granulocytopenia, a combination of LCF with casein or with egg white was quite effective. Casein, treated in formic acid solution with hydrogen peroxide to destroy methionine and tryptophane (7), was ineffective unless supplemented with both methionine and tryptophane. Dried human plasma was effective only after isoleucine was added. Zein and gelatin were ineffective. Supplementation of zein with tryptophane, lysine, histidine, and valine and supplementation of gelatin with tryptophane were also ineffective.

*Effects of Amino Acid Mixtures on Granulocyte Production*—The data in Table II indicate that LCF combined with a mixture of the ten "essential" amino acids was effective in correcting granulocytopenia. When arginine was omitted from the diet, six of eleven rats reached granulocyte levels of 2000 cells or greater. Omission of any other of the remaining nine essential amino acids, however, resulted in failure to produce significant granulocyte responses in 86 of 90 rats. Those four animals of the latter group which did show favorable responses lost weight, and only one survived 4 days beyond the recount. It may be noted that when the dietary level of essential amino acids (natural form) was raised from 8.7 to 18.0 per cent the survival was profoundly reduced.

*Effects of Various Proteins and Amino Acid Mixtures on Erythrocyte Production*—Routine determinations of hematocrit have been made in the studies of granulocyte production. The average hematocrit value at the time of detection (and treatment) of granulocytopenia was 32 volumes per cent. Distribution of the 512 individual values was as follows: >40 volumes per cent, 13.3 per cent; 39 to 30 volumes per cent, 55.4 per cent; 29 to 20 volumes per cent, 28.5 per cent; <20 volumes per cent, 2.7 per cent. As was noted previously, longer observation periods are necessary for studying the regeneration of erythrocytes as compared with the regeneration of granulocytes (9). The poor survival of granulocytopenic rats with

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note to Table II. Sodium bicarbonate was included in the diets (in place of equal weights of dextrose) to neutralize the hydrochlorides of the dibasic acids.

<sup>4</sup> Administered at a dietary level of 28.8 per cent to correct for non-protein constituents. This material, unfit for clinical use, was furnished by the Biologics Control Laboratory through the courtesy of Dr. M. V. Veldee.

inadequate treatment did not permit data to be obtained on the effect of some proteins and amino acid mixtures on erythrocyte regeneration. The

TABLE I  
*Effect of Various Proteins on Production of Granulocytes*

Treatment*	Amino acids	No. of rats treated	No. of rats surviving after 4 days	No. of rats with significant granulocyte responses	Polymorphonuclear granulocytes		Total white blood cells		Per cent of surviving rats with significant granulocyte responses
					Average values		Average values		
					Before treatment	After 4 days treatment	Before treatment	After 4 days treatment	
					cells per c.mm.	cells per c.mm.	cells per c.mm.	cells per c.mm.	
LCF		29	18	0	316	936	3980	4,097	0
Casein 18%		4	3	0	200	17	5767	1,350	0
"    30%		6	6	0	267	67	3883	1,983	0
"    50%		6	4	0	413	575	3438	2,550	0
LCF + casein 18%		13	11	7	359	2836	4036	10,482	64
" + "    30%		9	7	6	393	4078	4457	10,657	86
" + "    50%		17	7	5	257	2864	3143	8,293	71
" + oxidized casein		22	8	0	362	912	5131	3,792	0
"    "	Methionine	19	9	1	350	1061	4421	3,594	11
"    "	Tryptophane	15	6	1	317	1200	5682	4,766	17
"    "	Methionine + tryptophane	16	13	10	296	2969	3911	8,946	77
LCF	Same	6	5	0	380	590	3740	2,960	0
" + dried human plasma		5	5	0	330	560	6410	5,730	0
"    "	Isoleucine	8	4	4	250	6113	6350	19,125	100
LCF + zein		20	9	0	305	1061	2533	3,144	0
" + "	Tryptophane	8	4	0	212	950	4800	4,950	0
" + "	Tryptophane + lysine	8	6	1	266	1000	2809	3,250	17
" + "	Tryptophane + lysine + histidine	7	7	0	314	835	4114	5,435	0
" + "	Tryptophane + lysine + histidine + valine	9	4	0	250	1175	4225	5,650	0
" + gelatin		12	9	0	305	733	4177	3,822	0
" + "	Tryptophane	7	7	1	364	1450	3371	3,936	14
" + "	Tryptophane + methionine	9	1	1					
" + egg white		11	9	6	322	4822	4417	10,977	67

TABLE I—*Concluded*

\* *Lactobacillus casei* factor (LCF) (100  $\gamma$ ) was administered daily by pipette. Proteins were incorporated as 18 per cent of the diet except as otherwise indicated. Amino acids were incorporated in the diet in the amounts used in amino acid Mixture I (see foot-note to Table II). The combined weight of the natural amino acids and protein made up 18 per cent of the diet.

† A response was considered "significant" in these experiments when the polymorphonuclear granulocyte recount exceeded 2000 cells per c.mm.

effects of proteins and amino acid mixtures for which data were obtainable are presented in Table III. These data indicate that, as with granulocyte production, LCF or casein administered singly was ineffective, while their combined administration was effective. Oxidized casein promoted erythrocyte production only when supplemented with both methionine and tryptophane. Casein was replaceable to a large extent by a mixture of ten essential amino acids.

*General Findings*—Edema was noted in six rats on the protein-free basal diet after 21 to 25 days. In twenty-five rats edema was observed 1 to 9 days (average of 3 days) after the therapeutic administration of diets containing incomplete amino acid mixtures (Table II). Nine of these twenty-five rats were fed the threonine-free diet and seven the methionine-free diet. The edema was most prominent in the anterior thoracic region where it was noted in all but a few of the animals. A few rats had swollen fore limbs.

Serum protein determinations were made by a specific gravity method (10). An average concentration of 3.3 gm. per cent (range, 2.8 to 4.0) was obtained for nine rats after 18 days on the protein-free, basal diet as compared with an average value of 5.8 gm. per cent (range, 5.4 to 6.2) for ten rats fed an 18 per cent casein-containing diet for the same period.

Low protein intake has been shown to depress the liver storage of riboflavin and nicotinic acid (11). Therefore, some tests were made to determine whether these or other vitamins might influence the favorable granulocyte responses of these protein-depleted rats to therapeutically administered casein. Four granulocytopenic rats were treated with LCF and an 18 per cent casein-containing diet from which all vitamins were excluded. Pretreatment granulocyte levels of 200, 200, 300, and 500 cells per c.mm. increased after 4 days treatment to levels of 3050, 3150, 3100, and 10,550 cells per c.mm. respectively. Thus under these particular conditions the absence of vitamins did not impair the production of granulocytes.

Leucopenia was noted in most protein-depleted rats. Average values were about 4000 cells per c.mm. (Tables I and II). The responses of the total white blood cell count to treatment with various proteins and amino



TABLE II

*Effects of Various Amino Acid Mixtures on Production of Granulocytes*

<i>Lactobacillus casei</i> factor (100 $\gamma$ daily) combined with amino acids	No. of rats treated	No. of rats sur- viving after 4 days	Polymorphonuclear granulocytes		Total white blood cells		Per cent of surviving rats with significant* granulocyte responses	
			No. of rats with signifi- cant* granu- lyocyte re- sponse	Average values		Average values		
				Before treat- ment	After 4 days treat- ment	Before treat- ment		After 4 days treat- ment
				cells per c.mm.	cells per c.mm.	cells per c.mm.	cells per c.mm.	
Mixture I†.....	12	12	10	271	3091	3354	9,350	83
“ II‡.....	21	16	13	366	4181	3372	11,303	81
“ III§... .	17	5	2	290	2710	3860	7,380	40
“ II less arginine..	16	11	6	250	2254	3195	7,814	55
“ “ “ histidine..	15	12	0	304	433	3675	2,650	0
“ “ “ isoleucine..	20	12	1	350	654	4179	2,162	8
“ “ “ leucine..	16	8	0	306	644	3769	2,319	0
“ “ “ lysine...	17	10	0	340	575	4520	3,725	0
“ “ “ methionine..	20	9	0	311	750	3806	2,739	0
“ “ “ phenylala- nine. . . . .	20	8	1	312	1156	6081	4,056	13
Mixture II less threonine..	30	13	1	262	665	3738	2,792	8
“ “ “ trypto- phane.. . . .	20	11	1	291	954	4336	5,790	9
Mixture II less valine ...	17	7	0	336	79	4621	2,636	0

\* A response was considered “significant” in these experiments when the polymorphonuclear granulocyte recount exceeded 2000 cells per c.mm.

† Amino acid Mixture I was incorporated as 24.02 per cent of the diet and consisted of *l*-arginine monohydrochloride 1.14 gm., *l*-histidine monohydrochloride 0.61 gm., *dl*-isoleucine 1.44 gm., *l*-leucine 1.62 gm., *l*-lysine monohydrochloride 1.72 gm., *dl*-methionine 0.63 gm., *dl*-phenylalanine 1.40 gm., *dl*-threonine 1.40 gm., *l*-tryptophane 0.41 gm., *dl*-valine 2.88 gm., *dl*-aspartic acid 1.40 gm., *dl*-alanine 0.68 gm., *l*-cystine 0.23 gm., *l*-glutamic acid 3.96 gm., glycine 0.54 gm., *l*-hydroxyproline 0.36 gm., *dl*-norleucine 0.45 gm., *l*-proline 1.44 gm., *dl*-serine 0.54 gm., *l*-tyrosine 1.17 gm. In 24.02 gm. of these twenty acids were 18.0 gm. of free, natural acids. This mixture is No. XII-c of Rose and Fierke (8) with slight modifications (5).

‡ Amino acid Mixture II was incorporated as 13.25 per cent of the diet and consisted of the first ten amino acids (“essential” amino acids) enumerated in the composition of Mixture I in the amounts indicated above. In 13.25 gm. of these ten acids were 8.70 gm. of free, natural acids.

§ Amino acid Mixture III was incorporated as 33.18 per cent of the diet, and consisted of the first ten amino acids enumerated in the composition of Mixture I in the same relative concentrations. In 33.18 gm. of these ten acids were 18.1 gm. of free, natural acids.

acid mixtures were qualitatively similar to those of the granulocyte count. However, the total white blood cell count increased by only 2- to 3-fold under favorable treatment, while the granulocyte responses were usually greater than 10-fold under similar treatment.

TABLE III

*Effects of Various Proteins and Amino Acid Mixtures on Production of Erythrocytes*

Treatment*	No. of rats treated	No. of rats surviving after 8 days	Hematocrit values (average and range)		
			Before treatment	After 8 days treatment	Per cent change in average values
			vol. per cent	vol. per cent	
LCF†.....	29	9	35 (27-41)	23 (17-33)	-34
Casein 18-50%.....	16	3	30 (26-39)	22 (14-35)	-27
LCF + casein 18%.....	13	11	30 (21-38)	39 (29-48)	+30
“ + “ 30%.....	9	6	34 (30-39)	45 (34-52)	+32
“ + “ 50%.....	17	6	34 (29-40)	47 (44-49)	+38
“ + oxidized casein.....	22	4	36 (31-40)	28 (18-34)	-22
“ + “ “.....					
+ methionine.....	19	3	36 (33-38)	26 (21-29)	-28
LCF + oxidized casein					
+ tryptophane.....	15	5	36 (30-43)	28 (14-35)	-22
LCF + oxidized casein					
+ methionine + trypto-					
phane.....	16	12	33 (21-42)	37 (32-43)	+12
LCF + methionine + tryptophane.....	6	3	39 (33-42)	25 (20-31)	-36
LCF + egg white.....	11	9	33 (25-42)	39 (26-49)	+18
“ + amino acid Mixture					
I.....	12	12	34 (28-42)	39 (34-47)	+15
LCF + amino acid Mixture					
II.....	21	14	34 (24-43)	38 (32-44)	+12

\* These data are from the same rats which were treated for granulocytopenia under conditions indicated in Tables I and II.

† *Lactobacillus casei* factor.

Proteins and amino acid mixtures which promoted an effective increase in granulocytes were generally accompanied by gains in weight of 1 to 4 gm. per day. Significant weight gains also occurred in some rats (*i.e.*, treated with casein but not with LCF) in which granulocytes failed to form. Weight loss or failure to gain in weight was noted in most rats in which granulocyte responses were poor.

Failure to survive or poor granulocyte responses could not be attributed to the quantity of food ingested. For example, the average daily food

intake of rats fed amino acid-deficient diets (Table II) was 3.0 gm. for those which survived 4 days treatment and 3.1 gm. for those that did not. No clear correlations could be made between food intake and granulocyte responses of individual rats. Thus the four rats with favorable responses to amino acid-deficient diets (not including the arginine-free diet) (Table II) had average daily food intakes of only 1.2 to 2.8 gm., while thirty-four of the 86 rats which failed to respond had average intakes of 3.0 gm. or more.

#### DISCUSSION

The amino acid requirements for the formation of polymorphonuclear granulocytes as determined here in protein-depleted rats fall in line with the classic studies of Rose and his associates (12) on the amino acid requirements of rats for body growth. Robscheit-Robbins, Miller, and Whipple (13) have shown in the dog that a mixture of the ten essential amino acids and glycine favors new hemoglobin formation. Our preliminary findings in the rat have indicated too that a mixture of the ten essential acids is fairly effective in replacing casein in erythrocyte formation. Orten and Orten (14) have administered fifteen amino acids singly to anemic, protein-deficient rats and concluded that "no single amino acid can be regarded as a 'key' amino acid in hemoglobin synthesis." Further evidence is provided by studies of isolated deficiencies in rats of lysine (15), tryptophane (16), phenylalanine (17), and isoleucine (18), which indicate that each of these acids may be made a critical element in the formation of erythrocytes. Madden, Anderson, Donovan, and Whipple (19) reported that the ten essential amino acids plus glycine maintain nitrogen balance and promote plasma protein production in dogs. Thus it appears that the bulk of available evidence on the formation of specific proteins is in good agreement with Rose's studies on general tissue growth. This does not preclude the possibility that certain special conditions may require some "non-essential" amino acids.

The results with deficient proteins and amino acid supplements substantiate the findings with mixtures of amino acids. In addition, these results offer confirmation of the work of Toennies and Bennett (7, 20) on oxidized casein and of Orten, Bourque, and Orten (18) on the deficiency of isoleucine in blood proteins. However, the failure to make zein a suitable protein for granulocyte production even after supplementation with several amino acids, including tryptophane and lysine, is not in agreement with the work of Osborne and Mendel (21). The poor digestibility of zein and the depleted state of our rats may possibly be related to the differences in results.

Certain observations in the present studies indicate the relative toxicity of some amino acid mixtures and of a high casein intake. Only one of

seventeen rats fed a mixture of the ten essential amino acids at an 18 per cent dietary level was alive after an 8 day period as compared with the survival of fourteen of twenty-one rats fed the same mixture at half this level. It was also noted that the survival of rats fed gelatin with methionine and tryptophane was poorer than when gelatin alone was fed. It is noteworthy too that when fed a 50 per cent casein-containing diet only seven of seventeen rats receiving LCF survived a 4 day period, while eleven of thirteen comparable rats fed an 18 per cent casein-containing diet were alive after a similar period. The toxicities noted in the present work may depend on the prolonged period of complete protein deprivation that preceded the therapeutic administration of these proteins and amino acid mixtures. The nature of the defect responsible for the marked susceptibility of these depleted animals is not clear. It is quite possible that the enzymic mechanism for the digestion, utilization, or elimination of proteins and amino acids may be seriously impaired.

The poor survival of granulocytopenic rats has limited the period of observation following the therapeutic administration of inadequate proteins or amino acid mixtures. While this does not seriously limit the study of granulocytes (optimal responses of which are obtainable in 4 days), it does prevent an adequate study of the formation of hemoglobin, erythrocytes, or plasma protein. Perhaps modification of the present technique by the early use of a depletion method, such as hemorrhage, followed by the administration of test proteins and amino acids may provide suitable animals for such study.

The extremely high incidence of "folic acid" deficiency in rats fed these protein-free or low protein (casein) diets (5) as compared with the very low incidence in rats fed 18 per cent casein-containing diets (1) is of great interest. Possibly related are the findings of Wright *et al.* (22) indicating the favorable effect of high casein diets on the growth of rats fed sulfasuxidine. The basis for these protein effects has not yet been determined. Factors to be considered include the "folic acid" content of casein (including forms which may not be determinable by current techniques), the possible effect of protein on the storage, utilization, and retention of "folic acid," and the reaction of intestinal flora to varying protein levels in the diet.

#### SUMMARY

1. The amino acid requirements for granulocyte production were determined on rats prepared from weaning on protein-free diets. A mixture of the ten "essential" amino acids (8.7 per cent of the diet) successfully replaced casein or egg white in the production of granulocytes. Of these ten acids, none was dispensable except arginine which appeared to be essential

in only about half of the animals. Findings with amino acid supplementation of oxidized casein and dried plasma substantiated findings with mixtures of purified amino acids.

2. In the production of erythrocytes, the data indicate that a mixture of the ten essential amino acids can largely replace casein. Oxidized casein promoted erythrocyte production only when supplemented with methionine and tryptophane.

3. Administration of the essential amino acid mixture at an 18 per cent level to the protein-depleted rats resulted in a high mortality.

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## FREE TRYPTOPHANE IN BLOOD AND URINE\*

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Several schemes have been proposed for the fate of tryptophane in living organisms (1, 2) but relatively little is known about the distribution of the free amino acid in the animal body, primarily because no analytical techniques suitable for the routine determination of small amounts have been available until recently. In 1913 Abderhalden (3) used colorimetric methods to demonstrate that tryptophane was present in deproteinized plasma. Somewhat later Cary and Meigs (4, 5) analyzed cattle plasma colorimetrically and found an average of 1.12 mg. of free tryptophane per 100 ml.; 25 ml. were needed for a single determination. The amounts in blood were stated to vary with diet. More recently Albanese and Frankston (6) have used a chemical method for determining tryptophane in human urine, but the results obtained were roughly 10 times as high as those obtained by other methods (7).

Many of the newer microbiological methods for the amino acids appear to be at least as specific as the chemical ones and much more sensitive. Dunn *et al.* (8) have used *Lactobacillus arabinosus* for the determination of tryptophane in protein-free filtrates of human plasma, while Hier and Bergeim (9) have analyzed similar filtrates from dog plasma for leucine, isoleucine, threonine, and valine. In this laboratory both *Lactobacillus arabinosus* and *Streptococcus faecalis* have been used for the determination of tryptophane in urine (7, 10). The present study deals with the apparent free tryptophane<sup>1</sup> in the blood and urine of rats and other animals, and with the variations that result from certain changes in the diet.

### Methods

*Care of Animals*—Weanling or adult albino rats were kept singly in screen bottom cages. Mice were kept in groups of three to five in similar cages. Food and water were given *ad libitum*, and the animals were

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<sup>1</sup> It is recognized that peptides or other compounds closely related to tryptophane may stimulate the growth of the organisms used. In the present discussion the term "tryptophane" refers to microbiologically available compounds of this class.

weighed at weekly intervals. The diets (Table I) contained 0 to 80 per cent of purified casein (12) or 20 per cent of fibrin, 4 per cent of salts (11), 5 per cent of corn oil (Mazola), and glucose (cerelose) or dextrin (cooked starch) to 100 per cent. 1 part of halibut liver oil was added to 1000 parts of the corn oil used. A synthetic vitamin mixture (Table I) was added at a constant level to all of the diets except one from which pyridoxine hydrochloride was omitted. Animals were maintained on these diets 3 to 5 weeks before collecting urine or drawing blood.

For the collection of urine, individual rats or groups of three to five mice were placed in a metabolism cage fitted with an outside feeder. The urine was collected under a small amount of toluene, and after 72 hours the

TABLE I  
*Composition of Diets*

	<i>per cent</i>
Purified casein (or fibrin) ...	0, 5, 10, 20, 45, 50, 80
Corn oil + 0.1% halibut liver oil . .	5
Wesson's salt mixture (11) ..	4
Cerelose or dextrin to	100
	<i>γ per gm</i>
Pyridoxine hydrochloride	0, 2 5
Thiamine chloride	3.0
Nicotinic acid	5.0
Calcium pantothenate	20.0
Riboflavin	6.0
Choline chloride	500.0
Inositol ..	500.0
p-Aminobenzoic acid	300.0
Cystine	1000.0
l(-)-Tryptophane	0, 4800

funnels were rinsed with distilled water until the volume of urine and washings was approximately 100 to 150 ml. per rat per day. This corresponds to about a 10-fold dilution of the original urine. The volumes were recorded and aliquots removed. After filtration the pH was adjusted to 7.0, and the samples stored at 5°. Rat urine may be stored at this temperature for 1 week without appreciable destruction of tryptophane.

Blood samples were withdrawn by heart puncture under light ether anesthesia into a 10 ml. syringe previously rinsed with a 2 per cent solution of sodium citrate. 4 to 8 ml. of blood were removed from each rat, the samples centrifuged within a half hour, and aliquots of the plasma diluted 6-fold with distilled water. Microbiological assays were then made within 36 hours. Hier and Bergeim (9) have shown that blood samples may be stored for as long as 30 days with little change in the content of leucine,

isoleucine, valine, or threonine. Similar results were obtained in this laboratory for tryptophane.

*Determination of Tryptophane*—The method used was essentially that reported previously (7, 13). *Lactobacillus arabinosus* was grown on a semi-synthetic medium free from tryptophane, and aliquots of diluted plasma or urine were added in amounts corresponding roughly to 1 to 10  $\gamma$  of tryptophane per 10 ml. of medium. The cultures were incubated for 72 hours at 37° and the acidity produced was measured by titration. Each sample of plasma or urine was added to the cultures at three different levels with duplicate tubes at each concentration, and a standard curve for *l*-tryptophane was run with each series. Known amounts of the amino acid were added periodically to aliquots of the blood or urine, with recoveries of the added tryptophane ranging from 100 to 103 per cent.

In preliminary tests with dog plasma, protein-free filtrates were prepared according to the method of Dunn *et al.* (8) and aliquots of the neutralized filtrate added to cultures of *Lactobacillus arabinosus*. The values for *l*-tryptophane obtained were somewhat lower than when diluted plasma was added directly, but the recoveries of added *l*-tryptophane also tended to be low, 89 to 97 per cent when the protein-free filtrate was used. Attempts were also made to determine the tryptophane in diluted whole blood. The values and recoveries obtained were considered satisfactory, but, because of color and turbidity, it was necessary to use a pH meter for the titrations.

## Results

*Free Tryptophane in Blood of Various Species*—Free tryptophane was encountered in the blood of all species tested (Table II), and the amounts in the plasma of normal animals on stock diets were found to vary from an average of 9.2  $\gamma$  per ml. in the cow to 21.0 in the mouse. Variations within species were usually about  $\pm 20$  per cent from the average, although variations of several hundred per cent can be produced within a species by altering the diet (see below). The question is therefore open whether species differ *per se* in the levels of plasma tryptophane, or whether the amounts in the blood merely reflect differences in the dietary intake of this amino acid. Incidentally, the value for cattle, 9.2  $\gamma$  per ml., is in fair agreement with the figure of 11.2 obtained by Cary and Meigs in 1928 by a chemical procedure.

*Effect of Diet on Blood Tryptophane*—The level of free tryptophane in the blood was found to vary with the amount in the diet (Table III). Young rats fed for 6 weeks on a 10 per cent casein diet contained 10.5  $\gamma$  of free tryptophane per ml. of plasma; those on 20 per cent of casein averaged 16.8  $\gamma$  per ml., while on 50 per cent of casein the average trypto-



phane content was 27.8  $\gamma$  per ml. Variations within groups were fairly large. They did not, however, appear to be due to temporary fasting; blood from rats that had received no food for 4 hours contained as much tryptophane as blood from fully fed rats.

TABLE II  
*Free Tryptophane in Blood of Various Species\**

	Portion used	Tryptophane	
		Range	Average†
		$\gamma$ per ml	$\gamma$ per ml
Dog	Protein-free filtrate	10.7-13.5	11.9
"	Dilute plasma	13.2-15.3	14.3
Rat	Protein-free filtrate	15.1-16.2	15.7
"	Dilute plasma	13.6-16.3	15.3
"	Whole blood	15.1-20.3	16.7
Sheep	Dilute plasma	7.9-13.2	10.6
Cow	" "	8.7- 9.6	9.2
Swine	" "	10.0-12.0	11.0
Mouse	" "	15.6-26.4	21.0

\* We are indebted to Messrs. R. R. Spitzer, A. L. Pope, and W. R. Ruegamer for most of these samples.

† Three to five analyses included in the averages.

TABLE III  
*Effect of Dietary Protein on Free Tryptophane in Plasma from Young Rats*

Diet	No of animals	Tryptophane per ml plasma	
		Range	Average
		$\gamma$	$\gamma$
10% casein .. .	10	5.8-15.6	10.5
20% " . . .	6	13.7-20.8	16.8
50% " . . .	11	19.3-38.6	27.8
20% fibrin .	6	20.5-35.2	27.6
10% casein + <i>l</i> -tryptophane $\cong$ 40% casein	6	18.0-48.6	36.5

The higher levels of free tryptophane in the blood seemed to depend upon the intake of tryptophane itself rather than upon the total amount of protein ingested; essentially the same blood levels resulted on a diet containing 20 per cent of fibrin as when 50 per cent of casein was fed. The tryptophane contents of the latter diets were very similar, 0.54 per cent<sup>2</sup> and 0.6 per cent<sup>2</sup> of the total diet, respectively, with a higher food

<sup>2</sup> Microbiological determinations indicated that casein contained 1.2 per cent of tryptophane and fibrin 2.7 per cent.

intake on the fibrin diet tending further to equalize the intakes of tryptophane.

The highest blood levels, 36.5  $\gamma$  per ml., were reached when 0.48 per cent of *l*-tryptophane was added to a diet containing 10 per cent of casein. This amount of tryptophane is equivalent to that in the "extra" 40 per cent of casein contained in the 50 per cent casein diet. Since the concentrations of tryptophane in the blood were higher when the extra tryptophane was fed as the free acid than when combined in casein, it would appear that the other amino acids in a protein exert some influence on the rate at which a given amino acid (in this case tryptophane) is absorbed and utilized.

Essentially similar experiments were performed with adult rats except that two additional groups were included, one which was fed no protein in the diet, and another in which casein constituted 80 per cent of the total. Plasma from adult rats fed a protein-free diet for 4 weeks contained 9.2  $\gamma$  of tryptophane per ml. (6.4 to 12.8); plasma from rats fed the 80 per cent casein diet contained 23  $\gamma$  per ml. (17.3 to 29.0). When intermediate percentages of casein were fed, intermediate concentrations of tryptophane were found in the blood.

*Total Tryptophane in Certain Tissues*—Tissues from both rats and mice were hydrolyzed for 5 days at 37° with a mixture of pancreatin and erepsin (14) and the tryptophane content of the digests determined as before (7). In contrast to the free tryptophane of the blood, which varied with diet, the total tryptophane in muscle and liver proved to be relatively insensitive to changes in dietary protein. Some typical results on the livers of young rats are as follows: total tryptophane per gm. of fresh liver averaged 2.3 mg. (1.6 to 3.1) on the 10 per cent casein diet, 2.9 mg. (2.2 to 3.9) on 50 per cent of casein, and 2.5 mg. (2.2 to 2.8) on 20 per cent of fibrin. The amounts in muscle were 2.2 (1.9 to 2.4), 2.2 (2.1 to 2.4), and 2.3 (2.2 to 2.4) mg. per gm. respectively on the three diets. Essentially the same amounts of tryptophane were present in the tissues whether vitamin B<sub>6</sub> was omitted from the diet, or was present in adequate amounts.

*Disappearance of Tryptophane from Blood*—Two groups of rats were used in these experiments. The first was fed a relatively complete 45 per cent casein diet (Table I) for 5 weeks after being weaned; the second was fed a similar diet from which the pyridoxine was omitted. The rats were then injected intraperitoneally with a solution containing 4 mg. of *l*-tryptophane per ml. 1 ml. of the solution was given per 100 gm. of body weight. 30 minutes later very high amounts of tryptophane were noted in the blood, 47.9 and 31.3  $\gamma$  per ml. of plasma in the two groups respectively (Table IV). Thereafter the concentrations of the amino acid decreased rapidly, reaching normal levels within 2 hours.

Prior to the injection, and at all times thereafter, there was less free tryptophane in the blood of the pyridoxine-deficient rats than in the normal controls. Nevertheless, the rate of removal of the excess tryptophane from the blood was essentially the same whether pyridoxine had been fed or not.

TABLE IV

*Disappearance of Injected Tryptophane from Rat Blood*

4  $\gamma$  of *l*-tryptophane intraperitoneally per 100 gm. of body weight.

Time after injection	Tryptophane per ml plasma	
	Normal rats	Vitamin B <sub>6</sub> deficient rats
	$\gamma$	$\gamma$
<i>hrs</i>		
Uninjected	17 (15-18)	13.4 (11-18)
$\frac{1}{2}$	47.9 (44-58)	31.3 (28-35)
1	22.5 (18-27)	14.2 (14-15)
2	15.3 (13-17)	10.7 (9.5-11.8)

TABLE V

*Effect of Dietary Protein upon Urinary Excretion of Tryptophane by Rat*

Diet	Tryptophane ingested per rat daily*		Tryptophane excreted per rat daily†		Per cent excreted of tryptophane ingested	
	Young	Adult	Young	Adult	Young	Adult
	mg	mg	mg.	mg.		
0% casein		0		0.016		
5% "		6.8		0.091		1.34
10% " (cerelose)	10.3	12.4	0.12	0.084	1.13	0.67
10% " (dextrin)	9.2	10.0	0.17	0.075	1.86	0.75
20% "	26.1	36.7	0.31	0.19	1.19	0.51
20% fibrin	60.6		0.27		0.45	
50% casein	59.6	62.5	0.48	0.45	0.80	0.72
80% "	101.6	132.3	0.81	0.41	0.80	0.31
50% " minus vitamin B <sub>6</sub>	21.6	26.1	0.19	0.14	0.86	0.56

\* Microbiological determinations indicated that casein contained 1.2 per cent of tryptophane and fibrin 2.7 per cent.

† Most of the determinations are averages for at least four rats; many represent averages for ten to fourteen animals.

This suggests that the primary reaction by which tryptophane is removed from blood is not impaired in the pyridoxine-deficient rat, in spite of the fact that the over-all metabolism of this amino acid is abnormal, as manifested by the appearance of xanthurenic acid in the urine (15).

*Urinary Excretion of Tryptophane*—The excretion of tryptophane was determined in three species, rat, mouse, and man, and the amounts excreted

in the urine were found to depend upon the amounts of tryptophane ingested. Adult rats on a protein-free diet excreted only 16  $\gamma$  of tryptophane daily (Table V), but as progressively more casein was incorporated into the diet, the excretion of tryptophane was increased correspondingly. The largest amount excreted, 813  $\gamma$  per day, was by a group of young rats that received an 80 per cent casein diet (Table V). Other variations in the diet such as a change in the carbohydrate or the omission of vitamin B<sub>6</sub> did not appear to affect the excretion of tryptophane directly, although indirect effects were sometimes observed due to a marked change in the consumption of food, and therefore of tryptophane. Thus, young rats fed 50 per cent of casein excreted 477  $\gamma$  of tryptophane daily when the diet was adequate in vitamin B<sub>6</sub> but only 186  $\gamma$  on a diet deficient in this vitamin. However, on the latter diet very little food was consumed, and the percentages of ingested tryptophane that appeared in the urine were virtually identical on the two diets, 0.80 and 0.86 per cent respectively. The over-all average excretion of tryptophane by the various groups of rats was 1 per cent, the range being 0.31 to 1.86 per cent of that ingested (Table V).

Expressed as percentages of the tryptophane ingested, mice excreted 5 to 10 times as much of the tryptophane as rats on comparable diets, and the amounts of tryptophane excreted by a 15 gm. mouse were often greater than those excreted by 100 to 300 gm. rats. Thus mice on 20 per cent of casein excreted an average of 350  $\gamma$  of tryptophane daily (Table VI) as compared to 311 and 187  $\gamma$  by young and old rats respectively (Table V) on the same diet. The percentages of tryptophane excreted by the mouse ranged from 4.3 by a pyridoxine-deficient group on 50 per cent of casein to 12.3 per cent by an adult group on 5 per cent of casein. However, most of the groups excreted about 5 per cent of that ingested, and the percentage excreted did not appear to be affected consistently by the level of protein in the diet or by the nature of the protein (casein *versus* fibrin), or by the absence of vitamin B<sub>6</sub>.

The large amounts of apparent free tryptophane in mouse urine and in particular the presence of "tryptophane" in the urine of animals on protein-free diets raised the question whether the method of analysis employed was sufficiently specific for the purpose intended. The production of acid by *Lactobacillus arabinosus* is increased by certain derivatives of tryptophane such as indole and anthranilic acid (16, 17) even in the absence of tryptophane itself. Conceivably, therefore, the large amounts of "tryptophane" in urine might consist in part of other substances. However, mouse urine extracted with ether yielded essentially the same values for tryptophane as did unextracted urine. This procedure effectively removes indole (6, 17). Furthermore, the values obtained with *Lactobacillus arabinosus* could be duplicated with *Streptococcus faecalis*, which does not respond to either

indole or anthranilic acid (16). Accordingly, the tryptophane excreted on protein-free diets is assumed to come from the tissues and the observed difference in tryptophane excretion between the rat and mouse is also regarded as real. This difference parallels the previous observation that the pyridoxine-deficient mouse excretes a higher percentage of ingested tryptophane as xanthurenic acid than does the pyridoxine-deficient rat and further that increased amounts of dietary protein or tryptophane are particularly deleterious to the deficient mouse (12, 18).

TABLE VI

*Effect of Dietary Protein upon Urinary Excretion of Tryptophane by Mouse*

Diet	Tryptophane ingested per mouse daily	Tryptophane excreted per mouse daily	Per cent excreted of tryptophane consumed	No. of urinary collections*
	mg.	mg.		
10% casein	1.9 (1.6-2.8)	0.1 (0.055-0.14)	5.09 (2.0-8.7)	4
20% "	5.8 (5.8-5.9)	0.35 (0.14-0.57)	6.12 (2.5-9.7)	4
50% "	10.5 (10.0-12.3)	0.59 (0.24-0.83)	5.93 (2.2-8.3)	4
20% fibrin	12.2 (9.7-17.7)	0.54 (0.25-1.0)	4.43 (2.6-9.4)	4
5% casein (adults)	1.4	0.18	12.27	1
10% " minus vitamin B <sub>6</sub>	2.1	0.20	9.45	1
50% casein minus vitamin B <sub>6</sub>	6.7 (5.0-7.7)	0.29 (0.12-0.5)	4.3 (2.5-6.6)	3

\* Three to five mice per collection.

*Human Urine*—Preliminary analyses for tryptophane were made on urines<sup>3</sup> from college women subsisting on diets that supplied 30 gm. of protein daily as either whole eggs or soy beans. The urinary tryptophane per day averaged 6.9 mg. (5.5 to 9.8) which accounted for 1.5 per cent of the tryptophane ingested. Thus, as far as tryptophane excretion is concerned, human beings would appear to resemble the rat more closely than the mouse.

#### SUMMARY

1. A microbiological assay revealed that the free tryptophane in blood varied from 9.2  $\gamma$  per ml. of cow plasma to 21  $\gamma$  per ml. of mouse plasma. Plasma from the rat, dog, pig, and sheep yielded intermediate values.

<sup>3</sup> For these specimens we are indebted to Professor May Reynolds of the Department of Home Economics and her 1946 diet squad.

2. The apparent free tryptophane in rat plasma increased with increasing percentages of protein in the diet, from 9.2  $\gamma$  per ml. on a diet free from protein to 23.3  $\gamma$  per ml. on a diet containing 80 per cent of casein. The addition of *l*-tryptophane to the diet likewise increased the amount in the blood.

3. When *l*-tryptophane was injected intraperitoneally into rats, the levels in the blood ranged from 31 to 48  $\gamma$  per ml., but returned to normal concentrations after 2 hours. The rate of removal of tryptophane from blood was essentially the same in rats depleted of pyridoxine as in control animals receiving the vitamin.

4. The amount of tryptophane in the urine was found to depend upon the amount in the diet. Roughly 1 per cent of the tryptophane ingested by rats was excreted as such in the urine. Human beings excreted 1.5 per cent, while mice excreted from 4 to 12 per cent of that ingested.

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# THE SOLUBILITY CURVE AND THE PURITY OF INSULIN

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A method suitable for determining the degree of purity of insulin, which avoids the use of animals, would be a great help both for research and in the large scale production of the hormone.

Of the many methods proposed not one has been universally recognized as equal in value to the biological assay, but the latter is so cumbersome, time-consuming, and inaccurate that the advantage of a quicker and still reliable method is beyond question. Kunitz and Northrop (1) were the first to adopt the extensive use of the solubility curve as a criterion of purity for proteins, a method originally used by Sørensen and Høyrup (2), Landsteiner and Heidelberger (3), and Bonot (4).

The demonstration of the purity of the protein by its solubility curve is based on Gibbs' phase rule. With increasing quantities of solid phase present a pure protein will show a constant solubility, while a solubility varying with the amount of solid phase present is a clear indication of impurity.

Kunitz and Northrop (1) give a method of calculating the composition of the system if the protein is not pure. This method has not yet been applied to the determination of the purity of insulin and it appeared valuable to us to see whether it might be of any use for this purpose. As is shown in this paper, the method is unreliable and cannot replace the biological assay in the case of insulin.

## EXPERIMENTAL

All insulin used in this investigation was crystalline and prepared from beef pancreas in our own laboratories. The biological standardizations were carried out on rabbits. 60 to 80 animals were used for the standardization of each sample.

The conditions for determining the solubility curve are as follows: The solubility of the protein under investigation should not be too high nor too low in the given medium, since an unduly high solubility requires large quantities of material and makes the test rather expensive, while, if the solubility is too low, the determination of the amount dissolved may be difficult.

Preferably, the medium should be such that it does not interfere with the analysis of the dissolved fraction. The pH of the solution should be easily



reproducible and quite constant over a range of dilutions, since the solubility depends on it.

A medium should be chosen so that the effect of temperature on the solubility is at a minimum. Equilibrium should be reached within a reasonably short time to minimize appreciable deterioration of the protein during the experiment.

We found all of these conditions fulfilled to a large extent with insulin at its isoelectric point in a sodium acetate-acetic acid buffer solution. This buffer not only fixes the pH at the desired value but also makes the results independent of the small amounts of electrolyte possibly present as impurities in the insulin samples under investigation, which would otherwise interfere by increasing the solubility. The determination of the amount of protein in solution can be made by a simple micro-Kjeldahl analysis of the filtrate.

We found the following technique convenient and suitable for routine work. 75 mg. of the insulin are dissolved in 10 ml. of 0.1 N acetic acid,<sup>1</sup> and to this solution are added 20 ml. of 0.1 N sodium acetate, giving a buffer solution of pH 4.95. Varying amounts of the suspension thus obtained are added to a series of 50 ml. Erlenmeyer flasks, each containing 25 ml. of a buffer solution of the same composition. The amounts of the suspension added were usually 0.5, 1, 2, 4, and 8 ml.

After an hour to attain equilibrium the suspensions are filtered through an ashless filter paper of 6 cm. diameter, the filtrate being filtered through the same paper repeatedly until it becomes clear. Micro-Kjeldahl analyses are then made of the filtrates and of the original suspension.

At first sight the time to establish equilibrium seems to be surprisingly short, but experimental evidence shows that it is sufficient. A run was made with five solutions of exactly the same composition, each one containing 25 ml. of buffer and 4 ml. of the suspension of a crystalline insulin powder. They were filtered and analyzed after equilibration times of 1, 3, 7, 23, and 31 hours; the filtrates contained in all but the last instance 0.019 mg. of N per ml. After 31 hours we found 0.020 mg. of N per ml., an insignificant difference. The temperature has not much influence on the solubility, since a clear filtrate does not become cloudy when cooled from room temperature to the freezing point. Since it was not our intention to measure absolute values, we did not particularly fix the temperature by means of a thermostat. As all the solutions of one run were made simultaneously, they always were all at room temperature and a comparison within one run is justified.

The choice of the pH is of great importance. Not only the level of the

<sup>1</sup> Crystalline insulin usually does not dissolve quickly in this solution; sometimes it takes as much as 2 hours.

curve is dependent on the pH but also its slope, the latter being essential for the determination of the percentage composition. We chose pH 4.95 arbitrarily, because the solubilities at this pH have about the desired value and because a suspension of insulin crystals in water gives roughly the same pH.

In Fig. 1, *a* and *b*, the curves are given for the solubilities of two samples of the same insulin estimated at pH 4.95 and in a phosphate buffer of pH 6.0. In a phosphate buffer of pH 7.4 the insulin is completely in solution at all concentrations investigated.

The calculation of the percentage composition depends on the type of the precipitate. If the components form a solid solution, the calculation of the

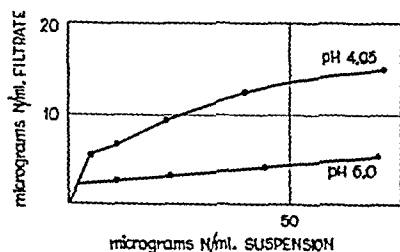
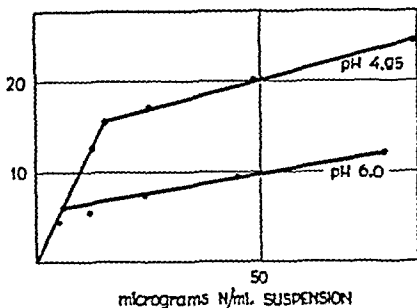
FIG. 1, *a*FIG. 1, *b*

FIG. 1. Solubility curves of insulin at pH 4.95 and pH 6.0: (*a*) Batch 148, (*b*) Batch h 269.

composition becomes impossible, since then the solubility of all of the components is involved in the formula. Only if the components are a mixture is it possible to calculate the purity of the insulin preparations.

Fig. 2 represents a theoretical solubility curve in the latter case with only one impurity present. Assuming insulin to be the less soluble component of the two, at *B* the solution is saturated with insulin.

A suspension with the quantity *AF* per ml. contains accordingly the quantity *EC* impurities and *AF - EC* of insulin. Assuming that 1 i.u. equals 45  $\gamma$  of pure insulin, the activity of the insulin sample can be expressed as  $45AF/(AF - EC)$   $\gamma$  per i.u. if the preparation is completely dry (1  $\gamma$  = 0.001 mg.).

If it contains *a* per cent moisture, the result becomes 1 i.u. =  $4500AF/(100 - a)(AF - EC)$   $\gamma$ . The value of 45  $\gamma$  for 1 i.u. of pure insulin will not be far from the truth and as a first approximation it will be sufficiently reliable. We have the impression, however, that the activity of absolutely pure insulin might be slightly greater, 1 i.u. in about 40  $\gamma$ .

### Results

An absolutely pure sample of insulin should give a completely horizontal solubility curve. Of the scores of samples investigated we found only one, that obtained without any special precautions by the method of Roman, Scott, and Fisher (5), to be absolutely pure.

The solubility curve was at a very low level, the filtrates containing as little as 0.005 mg. of N per ml., and the suspension up to 0.08 mg. of N per ml. The biological activity of this particular batch was about 40  $\gamma$  per unit.

Owing to lack of material, we were able to standardize it on only twenty rabbits. We went to considerable trouble to reproduce this result but without success. We recrystallized some batches of rather well shaped crystals seven to ten times but, as judged by the slope of the solubility curves, they did not much improve by this treatment. Anyhow this one

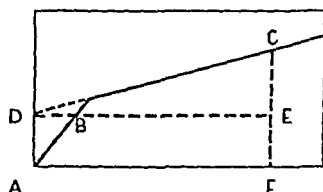


FIG. 2. Theoretical solubility curve for insulin in the presence of a single impurity. The abscissa represents the amount of insulin per ml. added; the ordinate, the amount per ml. dissolved; solid line, curve for the impure batch; *ABE*, curve for pure insulin; *AD*, solubility of pure insulin.

favorable result demonstrates that the possibility of obtaining a horizontal solubility curve is not a purely theoretical one.

With impure samples the question of solid solution or mixture must be solved first. If it is a mixture, the concentration of insulin in the filtrate should be independent of the total quantity present; if it is a solid solution, the concentration varies according to Raoult's law. In two instances we found by standardization of the filtrates on rabbits that the concentration of insulin dissolved in the filtrates is roughly proportional to their nitrogen content. The ratio of the N content of the filtrates of two different suspensions of the same insulin was 1:1.67; the ratio of their biological activities was 1:1.7. In another instance we found for the N content a ratio of 1:2.0 and for the activities 1:2.5. The standardizations were performed on twenty rabbits each. These results therefore prove that insulin and the impurities form a solid solution.

It is thus impossible to determine the percentage composition by means of the solubility curve without knowledge of the solubilities of each of the components.

However, we observed the remarkable fact that in some instances, particularly for insulin prepared by the method of Gerlough and Bates (6) and crystallized from a phosphate buffer according to Scott (7), the system

TABLE I  
*Activity of Insulin Preparations*

Results obtained by biological assay and by calculation from the solubility curve. Insulin obtained by the method of Gerlough and Bates and crystallized from phosphate-buffer solution, or by the method of Roman, Scott, and Fisher.

Batch No.	Biological test	Chemical test	Ratio of chemical to biological value
Gerlough and Bates			
	$\gamma$ per unit	$\gamma$ per unit	
139	52	60	1.15
140	63	63	1.00
142	64	68	1.06
146	64	68	1.06
147	60	58	0.97
148	60	60	1.00
150	84	78	0.93
154	82	73	0.89
155	75	74	0.99
157	75	66	0.88
158	69	73	1.06
241	58	55	0.95
329	59	52	0.88
4B	45	50	1.11
Average.....			0.99
Standard error.....			0.09
Roman, Scott, and Fisher			
235	40	45	1.11
240	60	70	1.16
244	43	60	1.40
251	65	70	1.08
256	61	70	1.15
266	50	73	1.46
Average.....			1.23
Standard error.....			0.16

can be treated as if the solid phase was a mixture and not a solid solution. The solubility curve is practically straight in the investigated range; *i.e.*, up to 0.09 mg. of N per ml. The activity was calculated by the above formula with the quantity *EC* (Fig. 2) obtained by graphical means. The

calculation has, of course, no theoretical foundation whatsoever but in practice it gives a good approximation of the activity. The results, presented in Table I, are almost convincing of the value of the methods as a first approach for estimating the activity. However, for insulin prepared by another method this rule of thumb failed completely and accordingly the usefulness becomes very doubtful for samples of unknown origin. In Table I are also summarized the results of our experiments with samples obtained by the method of preparation of Roman, Scott, and Fisher. Apparently the nature of the impurities is somewhat different from that of Gerlough and Bates' preparations and this has a profound influence on the slope of the solubility curve which, by the way, again approaches a straight line.

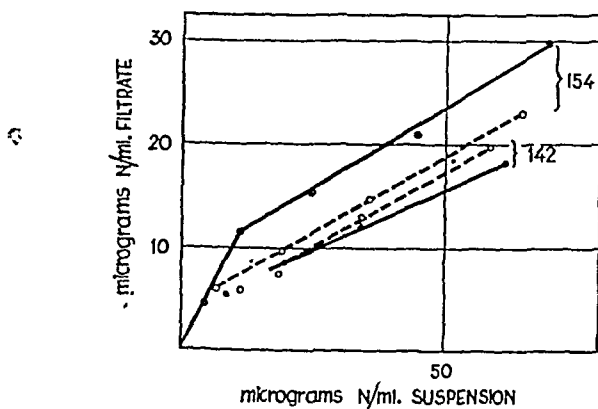


FIG. 3. Solubility curves of Batches 142 and 154 of insulin before (solid line) and after (broken line) alkali inactivation.

We can add another example of the limited value of the method even with crystalline insulin by the process of Gerlough and Bates. According to Freudenberg *et al.* (8) insulin can be completely inactivated by keeping it for 5 hours at 37° in 1/30 N NaOH. Considerable quantities of hydrogen sulfide and ammonia are liberated during this treatment, but the slope of the solubility curve of the inactivated product is not appreciably changed. This is demonstrated in Fig. 3 for two different insulin samples.

It is very unlikely that by treatment with alkali a single chemical entity results; it is much more probable that there will be more than one decomposition product. Still, so long as this has not been proved, there is a valid objection to any conclusion drawn from these experiments. If we suppose that all of the insulin has been decomposed to one and only one other substance, the percentage purity of the decomposition product will be equal to the percentage purity of the insulin in the original sample.

Therefore samples which are only partly decomposed are of particular interest. We obtained these samples by mixing, in different proportions, an original sample with a fully decomposed one. The suspension of the decomposed insulin contained 0.047 mg. of N per ml., of the original 0.077 mg. of N per ml. With the acetate buffer of pH 4.95, mixtures of these two suspensions were made and the filtrates analyzed for N content. The data obtained show that substitution of insulin by its alkali decomposition product does not alter the total solubility appreciably. This (see Table II) is another indication that we are dealing with a solid solution. Thus

TABLE II  
*Solubility of Mixtures of Crystalline Insulin and Alkali-Inactivated Insulin at pH 4.95*

Insulin <i>per cent</i>	Decomposition product <i>per cent</i>	Suspension <i>mg. N per ml.</i>	Filtrate	
			Found <i>mg. N per ml.</i>	Calculated <i>mg. N per ml.</i>
0	100	0.047	0.015	
35	65	0.050	0.019	0.018
62	38	0.060	0.020	0.020
82	18	0.068	0.021	0.022
100	0	0.077	0.024	

admixture of alkali-inactivated insulin with an active insulin sample is accordingly not demonstrable in this way.

Denatured insulin is much less soluble than native insulin and, since it is insoluble in 0.1 N acetic acid, its presence is readily detected. The solubility in the buffer of pH 4.95 is very low indeed. We found values between 0.002 and 0.004 mg. of N per ml. in the filtrate. This is not due to slow equilibration, since the values after 1 and 24 hours, 0.003 and 0.002 mg. per ml. respectively, were identical within experimental error. The stability of the denatured insulin under these circumstances is remarkable. We expected to see an increase of the solubility after 24 hours, owing to a shift in the equilibrium to the native product.

#### DISCUSSION

Provided a specimen of insulin is pure, the solubility curve is a valuable criterion for demonstrating its absolute purity and in fact the only proof at present available. It is apparently very sensitive. For an impure batch the solubility curve gives only a clear proof that it is *not* absolutely pure, but the amount of impurity cannot be derived from the solubility curve with a sufficient degree of certainty. This is due to the formation of solid solutions of the insulin and the impurities, which is more or less

to be expected here, since the main impurities of insulin crystals will consist of closely related proteins with properties so similar to insulin that removal during the purification process has not been effected, while other impurities may also consist of decomposition products of insulin.

There is still a remote possibility of obtaining a solubility curve of the mixed component type by altering the conditions for precipitation. Salt-ing-out or precipitation with alcohol seemed a likely method, but experiments carried out with sodium sulfate or ethyl alcohol gave unsatisfactory results.

As a routine test, instead of the ordinary biological assay for estimating the activity of insulin, the solubility curve seems unsuitable, but it remains of great value as a criterion for absolutely pure material.

It might be desirable to have an absolutely pure international standard, though possibly the difficulty of obtaining sufficiently large quantities outweighs the advantage of reproducibility. The solubility curve would be a useful guide in the selection of batches suitable for this purpose.

## O

## SUMMARY

The solubility curve of insulin samples in a sodium acetate-acetic acid buffer of pH 4.95 has been determined. This curve is of the solid solution type and it is impossible to calculate exactly the purity of the samples from the results, since the solubility of the impurities is unknown. We succeeded once in obtaining an absolutely pure sample of crystalline insulin with a solubility independent of the amount undissolved.

Occasionally the treatment of the system as being of the mixed crystal type gives a fair approximation to the percentage insulin of the sample but the method is unreliable and cannot be generally recommended.

The solubility curve is not appreciably altered by alkali inactivation of the insulin.

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# MICRODETERMINATION OF $\alpha$ - AND $\beta$ -GLYCEROPHOSPHATES\*

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During an investigation (1) of the nature of the  $\alpha$ - and  $\beta$ -phospholipide fractions reported by Suzuki, Yokoyama, and Nishimoto (3-6), it became desirable to analyze the  $\alpha$ - and  $\beta$ -glycerophosphates formed by the hydrolysis of these fractions. Micromethods were developed in which the combinations of the reactions described by Fleury and Paris (7), Bailly (8), and Fleury and Courtois (9) were used.  $\alpha$ -Glycerophosphate was converted to glycolic aldehyde phosphate by reaction with periodate at room temperature, the excess of periodate and the iodate formed were

\* The data in this paper are taken from a thesis which is being submitted to the Graduate School of The University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report of these data is given in (1). Before the final preparation of this paper for publication, a report appeared by Leva and Rapoport (2) which described methods for the determination of  $\alpha$ - and  $\beta$ -glycerophosphates in trichloroacetic acid extracts of liver tissue based upon the same reactions as those used in this paper for the determination of  $\alpha$ - and  $\beta$ -glycerophosphates in hydrolyzed phospholipides. In calculating their results, it was necessary for Leva and Rapoport to use correction factors, whereas the results obtained in this paper are theoretical. In the case of their  $\alpha$ -glycerophosphate P, they claimed that on pure barium  $\alpha$ -phosphoglycerol solutions their recovery was only 96.5 per cent. According to the literature and according to our work, the recovery is 100 per cent, and we can offer no explanation for this discrepancy. In the case of the sum of the  $\alpha$ - and  $\beta$ -glycerophosphate P, the difference in results can be explained by a difference in method. Leva and Rapoport determined this value by first heating the sample with acid for 1 hour, then oxidizing at room temperature with periodic acid for 1 hour, followed by a hydrolysis of the phosphoglycolic aldehyde for 1 hour after destruction of the periodate with bisulfite. Using this procedure, they reported an average recovery of only 86.5 per cent. In our work, we carried out the three steps simultaneously and obtained 100 per cent recovery in 1 hour's time. This is reasonable because, when the three steps are combined, the  $\alpha$ -glycerophosphate which is formed from the  $\beta$ -glycerophosphate immediately reacts with the periodic acid present and is removed from the field of action, thus shifting the equilibrium to the right and inducing a 100 per cent conversion of  $\beta$ -glycerophosphate to  $\alpha$ -glycerophosphate. When the conversion of  $\beta$ -glycerophosphate to  $\alpha$ -glycerophosphate is carried out as a separate step by heating with acid, an equilibrium mixture containing about 93 per cent  $\alpha$ -glycerophosphate is obtained. This fact plus the low recovery they obtained on  $\alpha$ -glycerophosphate would probably explain the correction factor of 86.5 per cent used by Leva and Rapoport. Thus the method as used by us for the sum of the  $\alpha$ - and  $\beta$ -glycerophosphate P is both simpler and more accurate.

destroyed with sodium sulfite, and the glycolic aldehyde phosphate was hydrolyzed with hot acid. The resulting orthophosphate was measured by a modification of a standard colorimetric procedure (10). When corrected for the inorganic phosphate produced by hydrolysis of phospholipide, the orthophosphate measured the  $\alpha$ -glycerophosphate or  $\alpha$ -phosphorus. Total glycerophosphate ( $\alpha + \beta$ ) was measured on another portion of the sample by converting all of the glycerophosphate to the  $\alpha$  form and then to orthophosphate by treating it with periodate in hot acid solution. The  $\beta$ -glycerophosphate was found by subtracting the  $\alpha$ -phosphorus value from the  $\alpha + \beta$ -phosphorus. Total phosphorus was determined by a modification of a procedure described by Gortner (11) and served as a check on the other determinations.

### Procedure

*Inorganic P*—A 5 cc. sample containing 0.01 to 0.05 mg. of phosphorus was pipetted into a 10 cc. glass-stoppered graduated cylinder, and 1 cc. of 4 per cent sodium sulfite, 1 cc. of 10 N sulfuric acid, and 1 cc. of 7 per cent sodium molybdate solution were added in succession from burettes. The contents of the cylinder were mixed by inverting, and 1 cc. of a dilute solution of stannous chloride<sup>1</sup> was added from a burette. The cylinder was filled to the 10 cc. mark with distilled water, stoppered, and inverted six times. After 20 minutes the intensity of the blue color was read on a Klett-Summerson photoelectric colorimeter with a No. 540 filter. A blank on the reagents was used to set the zero point of the instrument. Phosphorus standards for comparison were prepared by adding a solution of potassium dihydrogen phosphate containing 0.01 mg. of phosphorus per cc. to the reagents.

*$\alpha$ -Glycerophosphate P*—A 5 cc. sample containing 0.01 to 0.05 mg. of phosphorus was pipetted into a 1  $\times$  8 inch test-tube and allowed to react for 10 minutes at room temperature with 1 cc. of 0.05 M periodic acid in the presence of 1 cc. of 0.1 N sulfuric acid. 1 cc. of 4 per cent sodium sulfite solution and 1 cc. of 10 N sulfuric acid were then added and the test-tube immersed halfway in boiling water for 1 hour. The contents of the tube were cooled under tap water, poured into a 10 cc. graduated cylinder, and made up to the mark with the washings. After a half dozen inversions of the stoppered cylinder, a 5 cc. sample was pipetted out for the colorimetric measurement of orthophosphate, as described under the procedure for inorganic P, with the exception that only 0.5 cc. of additional 10 N sulfuric acid was needed. This procedure gives the  $\alpha$ -P + inorganic P.

<sup>1</sup> 40 gm. of c.p. stannous chloride were dissolved in 100 cc. of concentrated hydrochloric acid. 1 cc. of this solution was diluted to 200 cc. with distilled water just before use.

The inorganic P which has been determined on another portion of the sample is subtracted from this value to give the  $\alpha$ -P.

*Total ( $\alpha + \beta$ ) Glycerophosphate P*—A 5 cc. sample containing 0.01 to 0.05 mg. of phosphorus was pipetted into a 1  $\times$  8 inch test-tube containing 1 cc. of 10 N sulfuric acid and 1 cc. of 0.05 M periodic acid. The test-tube was half immersed in boiling water for 1 hour, during which time the volume was maintained between 5 and 7 cc. by addition of distilled water. The test-tube was cooled, 1 cc. of 4 per cent sodium sulfite solution was added, and the solution was transferred to a 10 cc. graduated cylinder and made up to the mark with the washings. As in the  $\alpha$ -P procedure, a 5 cc. aliquot was taken for colorimetric measurement of the orthophosphate produced and only 0.5 cc. of 10 N sulfuric acid was added.  $\alpha$ - +  $\beta$ - + inorganic P are measured by this procedure. The values for inorganic P and  $\alpha$ -P are subtracted to give the value of  $\beta$ -P.

*Total P*—A sample containing 0.01 to 0.05 mg. of phosphorus was pipetted into a 1  $\times$  8 inch Pyrex test-tube containing 1 cc. of 10 N sulfuric acid and a few glass beads. The solution was gently heated over a micro burner until it turned dark. It then was allowed to cool for a minute and 1 drop of superoxol was added. Gentle heating was resumed until the solution cleared and then the flame was increased until fumes of sulfuric anhydride appeared.<sup>2</sup> The digested solution was cooled, transferred to a 10 cc. graduated cylinder, and made up to volume. 5 cc. aliquots were taken for colorimetric phosphate determination with the addition of only 0.5 cc. of 10 N sulfuric acid instead of the 1 cc. used in the inorganic P procedure. It usually is not necessary to run this determination, since the total P has been found equal to the sum of the inorganic P + total glycerophosphate P. However, it serves as a check and might reveal the presence of an unknown phosphorus compound.

#### EXPERIMENTAL

*Comparison of  $\alpha$ -P Procedure with Standard Titrimetric Method*—A solution of  $\alpha$ -glycerophosphate was prepared by heating sodium  $\beta$ -glycerophosphate with strong acid, and the  $\alpha$ -phosphorus was determined by both the microcolorimetric method described in this paper and the titrimetric periodate procedure described by Fleury and Paris (7).

Sodium  $\beta$ -glycerophosphate (Eastman Kodak, No. 3532) was heated for 1 hour with 10 per cent sulfuric acid at the boiling point. The acid was neutralized and precipitated by stirring the solution for 12 hours with finely ground barium carbonate, and the barium sulfate and excess barium

<sup>2</sup> Gortner (11) eliminated the last traces of peroxide by dilution with water and bringing to fumes again. This step was found to be unnecessary when sodium sulfite was used in the subsequent colorimetric measurement of phosphate.

carbonate were removed by filtration. The filtrate and washings were combined in a volumetric flask, were made up to volume, and aliquots were taken for analysis. The titration of the periodate reduced by samples containing more than 1 mg. of  $\alpha$ -phosphorus was carried out with the 0.1 N solutions employed by Fleury and Paris (7). For samples containing 0.01 to 1 mg. of  $\alpha$ -phosphorus, 0.005 N periodic acid, 0.01 N sodium arsenite, and 0.003 N iodine solutions were used. Total phosphorus of the solution from which aliquots were taken for  $\alpha$ -phosphorus analysis was determined by

TABLE I  
*Comparative Analysis of  $\alpha$ -Phosphorus in Sodium  $\alpha$ -Glycerophosphate Solution by Microcolorimetric and Titrimetric Methods*

Total phosphorus in sample	Method for $\alpha$ -phosphorus	Periodate reduced	$\alpha$ -Phosphorus found by analysis	Phosphorus converted to $\alpha$ -phosphorus
mg.		micromoles	mg.	per cent
19.70	Macrotitrimetric	1162	18.02	91.6
7.88	"	473	7.35	93.3
3.94	"	235	3.65	92.6
3.94	"	236	3.67	93.2
3.94	"	236	3.67	93.2
0.878	Microtitrimetric	52.8	0.820	93.3
0.878	"	53.5	0.830	94.5
0.439	"	26.1	0.404	92.0
0.0878	"	5.26	0.0819	93.2
0.0439	"	2.58	0.0398	90.7
0.0439	"	2.61	0.0401	91.3
0.0410	Microcolorimetric		0.0386	94.1
0.0410	"		0.0385	94.0
0.0410	"		0.0383	93.4
0.0410	"		0.0379	92.5
0.0410	"		0.0386	94.2
0.0410	"		0.0383	93.4

the microcolorimetric procedure. The analytical results for  $\alpha$ -phosphorus and the per cent of phosphorus present as  $\alpha$ -phosphorus are shown in Table I. The values obtained by the microcolorimetric procedure agreed with those obtained by titration of both micro and macro samples. The per cent conversion was in agreement with the 93 per cent reported by Bailly (8).

*Analysis of Sodium Glycerophosphate by Microcolorimetric Procedure*—To test the procedures described in this paper, sodium glycerophosphate (Eastman Kodak, No. 644) was analyzed for inorganic P,  $\alpha$ -P, ( $\alpha + \beta$ )-P, and total P. The results are shown in Table II. The sum of the average values for inorganic P,  $\alpha$ -P, and  $\beta$ -P equaled the average value for total P,

yolk by the procedure of Yokoyama (6). The mixture was refluxed on the steam bath for 1 hour with 10 cc. of 0.1 N alcoholic sodium hydroxide. It then was cooled to room temperature, acidified with 0.1 N acid, and 20 cc. of additional water were added. The alcohol-water solution was extracted three times in a separatory funnel with 20 cc. portions of petroleum ether containing 5 per cent of chloroform. The water-soluble residue was made up to 100 cc. volume and 10 cc. aliquots were taken for inorganic P,  $\alpha$ -P,  $\beta$ -P, and total P. Another 35.88 mg. sample of egg lecithin without added sodium glycerophosphate was hydrolyzed, acidified, and extracted in the same manner, and then analyzed for the types of phosphorus mentioned. A comparison of the values obtained by addition of the individual analyses of the sodium glycerophosphate and of the hydrolyzed lecithin with those obtained after hydrolysis of the mixture is shown in Table III. The sum of the values determined by analyses of the separate samples did not differ significantly from the values found for the combination.

#### DISCUSSION

The titrimetric periodate procedure for  $\alpha$ -glycerophosphate described by Fleury and Paris (7) for use of 0.1 N solutions on macro samples was found to serve equally well with 0.005 or 0.01 N solutions on samples of sodium glycerophosphate containing 0.01 to 1 mg. of  $\alpha$ -phosphorus. However, ethanolamine, serine, and inositol are known to be present in the hydrolysate of phospholipids (13-15) and have been shown to react with periodate (16-18). For this reason, the titrimetric periodate methods cannot be used directly to measure  $\alpha$ -glycerophosphates in phospholipids.

An experimental study of the rate of reaction of periodate with  $\alpha$ -glycerophosphate showed that it must be carried out at a pH of 3 or less in order to secure quantitative results in 10 minutes at room temperature. In the procedure as given here, 0.1 N sulfuric acid was used to give a pH less than 3.0.

In analyzing for  $\alpha$ -glycerophosphate, the glycolic aldehyde phosphate produced by the action of periodate with  $\alpha$ -glycerophosphate is hydrolyzed by heating with 1 N sulfuric acid for 1 hour. It has been found that the aldehyde phosphate is completely hydrolyzed by this treatment and that any  $\beta$ -glycerophosphate present is not affected if the excess of periodate is first destroyed by use of sodium sulfite.

When the three reactions involved in the conversion of all of the glycerophosphate to orthophosphate, namely (a) conversion of the  $\beta$  isomer to the  $\alpha$  isomer, (b) reaction of the  $\alpha$  isomer with acid periodate, and (c) hydrolysis of the aldehyde phosphates, are carried out at the same time, not only is time saved but the conversion of  $\beta$  to  $\alpha$  is 100 per cent complete instead of the 93 per cent obtained when carried out as a separate step. The excess

of periodate and the iodate formed by its reduction caused fading of the blue color of the molybdenum-phosphate complex, and therefore had to be removed from the solution or destroyed. Treadwell and Hall (19) state that sulfite converts both periodate and iodate to iodide. Sodium sulfite was used therefore to destroy these oxidizing agents in the total glycerophosphate procedure after the hydrolysis of the aldehyde phosphate was completed.

A study of the effect of iodide and sulfite added to standard solutions of potassium dihydrogen phosphate showed that they did not interfere with the colorimetric measurement of orthophosphate when stannous chloride was used as the reducing agent. In fact, the sulfite stabilized the blue color so that the straight line relationship reached in 20 minutes between color intensity and phosphorus present still existed 24 hours later. Sodium sulfite was found also to destroy any traces of hydrogen peroxide left after digestion of the total phosphorus samples. For these reasons, the addition of 1 cc. of 4 per cent sodium sulfite solution was made an integral part of the colorimetric determination of orthophosphate in the inorganic P,  $\alpha$ -P, ( $\alpha + \beta$ )-P, and total P procedures. The amount of sulfite added had no effect on the color intensity; so that the sulfite left in the orthophosphate by the conversion of glycerophosphate did not cause error.

The amounts of sulfuric acid added to the samples for color measurement were varied in order to secure the same final concentration of acid in all procedures.

When these methods are applied to the hydrolysate from phospholipide, any phosphorus present as unsaponified phospholipide must be removed from the water solution because it was found to be almost quantitatively converted to orthophosphate by the  $\alpha$ - and  $\beta$ -phosphorus procedure. It was extracted more readily by petroleum ether containing 5 per cent of chloroform than by petroleum ether alone.

#### SUMMARY

The colorimetric measurement of the orthophosphate produced by the reaction of periodate on  $\alpha$ -glycerophosphate followed by acid hydrolysis has been shown to agree with the results obtained by titration of the periodate reduced.

Microcolorimetric methods for inorganic P,  $\alpha$ -P, ( $\alpha + \beta$ )-P, and total P have been described and shown to be suitable for the analysis of solutions of sodium glycerophosphate and of the phosphorus compounds found in the hydrolysate of a phospholipide.

The author wishes to acknowledge his great indebtedness to Dr. W. R. Bloor for making this work possible.

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# THE ACTION OF RIBONUCLEASE ON THE SUCCINOXIDASE SYSTEM

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In a previous paper from this laboratory, a crystalline preparation of ribonuclease was shown to inhibit succinic dehydrogenase in tissue homogenates (10), and it was suggested that ribonucleic acid might be in some way associated with the protein of succinic dehydrogenase. Such a possibility was also indicated by the work of Claude (5, 6) who found that the mitochondria of tissues showed high succinoxidase activity and contained ribonucleic acid.

Subsequent work by Cohen (7) showed that proteolytic activity was associated with crystalline ribonuclease either as an impurity or as an intrinsic property,<sup>1</sup> and this at once raised the question of whether the results of Potter and Albaum (10) may have been due to proteolytic destruction of succinic dehydrogenase rather than to any reaction involving the ribonucleic acid in the mitochondria. It was thus desirable to determine whether inhibition of succinic dehydrogenase was correlated with ribonuclease activity or with proteolytic activity, and by applying the author's methods (12) for the quantitative determination of nucleic acids, such a study has been made.

## Materials and Methods

*Measurements*—Succinic dehydrogenase was measured as described previously (13). Nucleic acids were extracted and determined by the method of Schneider (12).

*Tissue Preparations*—Homogenates of mouse and rat liver in ice-cold 0.0002 N NaOH (pH 9.5, the "neutral" water of Claude (4)) were made with the apparatus of Potter and Elvehjem (11). Mitochondria (large granules) were prepared from these homogenates by the method of Claude (4, 5) with the following modification. The sediment obtained by centrifuging the homogenate at  $1500 \times g$  was washed three times by resus-

\* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

<sup>1</sup> A personal communication from Dr. Kunitz calls attention to the possibility of traces of trypsin and of chymotrypsin as contaminants in crystalline ribonuclease.

pension in a volume of neutral water equivalent to one-fourth of the volume of the original homogenate, followed by centrifugation at the same speed. The washings were found necessary to minimize the loss of mitochondria in this sediment. The mitochondria were then sedimented from the combined supernatant and washings and after washing were suspended in enough water to bring the total volume to that of the original homogenate. Such a mitochondria suspension is called a 10 per cent suspension because the original homogenate from which the mitochondria were sedimented had a concentration of 10 per cent. As Claude has pointed out, a mitochondrion suspension prepared from liver probably contains secretory granules in addition to mitochondria (5).

*Enzyme Preparations*—Three crystalline preparations of ribonuclease were used in the present study. One of these was employed in the previous work (10) (Sample A). A second sample (B), also supplied by Dr. M. Kunitz, was obtained more recently. The third sample (C) was prepared in this laboratory by Mr. G. C. Mueller and was not entirely free of ammonium sulfate. The crystalline chymotrypsin used was the same sample as that employed previously (1).

### Results

*Effect of Ribonuclease on Isolated Mitochondria*—In this experiment the effect of ribonuclease on the succinoxidase activity and the pentose nucleic acid (PNA) content of rat liver mitochondria was determined. The results are reported in Fig. 1. It is apparent that the ribonuclease has a more striking and earlier effect on the PNA than on the succinoxidase activity. At the end of 100 minutes of incubation, the succinoxidase activity had been decreased only 35 per cent, while 85 per cent of the PNA had disappeared by the end of 20 minutes. Subsequent experiments with incubation periods shorter than 20 minutes showed that the PNA had disappeared to the maximum extent by the end of 2.5 minutes of incubation. It was never possible to remove the PNA completely from mitochondria with ribonuclease, and 10 or 15 per cent of the PNA always remained after the action of the crystalline enzyme. The slow loss of PNA observed in the control samples incubated in the absence of added ribonuclease may be due to tissue ribonuclease, since the enzyme has been shown to be associated with mitochondria (6). It would thus appear that the major portion of the PNA of mitochondria is not necessary for the oxidation of succinate and that the inhibition of succinoxidase by ribonuclease involves some mechanism other than removal of PNA.

*Effect of Ribonuclease on Mitochondria and Homogenate*—The inhibition of succinoxidase obtained in the first experiment (35 per cent) was considerably less than had been obtained previously (10). This was found

to be due to the fact that the incubation had been made with mitochondria rather than with the whole homogenate. The effect of ribonuclease on the succinoxidase activity of a mouse liver homogenate and of the mitochondria isolated from the homogenate was determined and the results are presented in Table I. The data clearly show that the homogenate is more strongly inhibited than are the mitochondria. The succinoxidase  $Q_{O_2}$  of isolated liver mitochondria is about 4 times as great as that of a

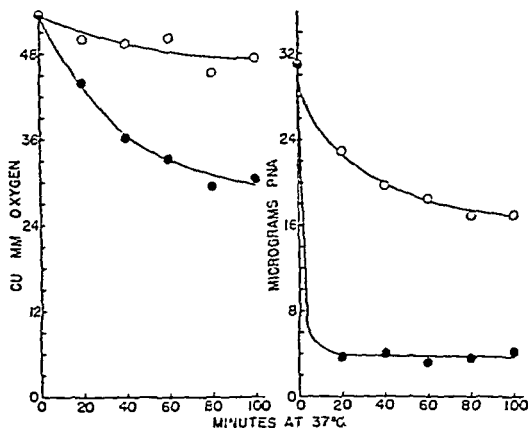


FIG. 1. The effect of the time of incubation with ribonuclease on the succinoxidase activity (c mm of  $O_2$  per 10 minutes per 0.2 ml of 10 per cent mitochondria) and the pentose nucleic acid (PNA) content (micrograms per 0.2 ml of 10 per cent mitochondria) of isolated rat liver mitochondria. The incubations were made as follows: 1.5 ml of 10 per cent mitochondria were mixed with 0.2 ml of 0.5 M sodium succinate (pH 7.4) and 1.5 ml of 0.2 per cent ribonuclease (Sample A) or 1.5 ml. of water. At the end of the incubation times samples were withdrawn for PNA and succinoxidase measurements. Open circles, mitochondria incubated with water; closed circles, mitochondria incubated with added ribonuclease.

liver homogenate (unpublished experiments), so that a 4-fold purification of the succinoxidase system has been accomplished by isolation of the mitochondria. One might predict that the purified system would be more sensitive to ribonuclease than the homogenate but actually the reverse was the case (Table I).

*Effect of Different Ribonuclease Samples*—Three different crystalline samples of ribonuclease were compared in their effect on the succinoxidase activity of mouse liver homogenates (Table II). Only Sample A showed significant inhibition of the succinoxidase system. This was the sample used in the previous study (10). It would thus seem that this sample

TABLE I

*Effect of Ribonuclease on Succinoxidase Activities of Isolated Mouse Liver Mitochondria and of Mouse Liver Homogenate*

The incubations were made in the side arms of Warburg flasks. The total volume of fluid in the side arm was 0.4 ml. and contained 0.1 ml. of 10 per cent mouse liver homogenate or 20 per cent mouse liver mitochondria, 0.05 ml. of 0.5 M sodium succinate (pH 7.4), and ribonuclease or water. The ribonuclease used was Sample A. At the end of 120 minutes incubation at 37°, the contents of the side arms were dumped into the main compartment of the flask containing the components of the succinoxidase system in a volume of 2.6 ml. (cf. (6)).

Tissue preparation*	Ribonuclease added	Oxygen uptake	Inhibition
	$\gamma$	c.mm. per 10 min.	per cent
Homogenate.....	0	47.6	
“.....	150	27.8	43
“.....	225	0	100
Mitochondria.....	0	64.7	
“.....	150	60.0	7
“.....	225	49.1	24

\* The oxygen uptakes for samples of homogenate and mitochondria which had been kept at 0° during the incubation period were 50.1 and 66.8 c.mm. per 10 minutes, respectively.

TABLE II

*Effect of Different Ribonuclease Samples and of Chymotrypsin on Succinoxidase Activity of Mouse Liver Homogenates*

The incubations were made in the side arms of Warburg flasks. The side arms contained 0.05 ml. of 0.5 M sodium succinate (pH 7.4), 0.1 ml. of 10 per cent mouse liver homogenate, and water or enzyme solutions to make the total volume 0.4 ml. At the end of 120 minutes incubation at 37°, the contents of the flasks were dumped into the main compartment of the flask containing the components of the succinoxidase system in a volume of 2.6 ml. (cf. (6)).

Crystalline enzyme added	Amount per flask	Oxygen uptake	Inhibition
	$\gamma$	c.mm. per 10 min.	per cent
None.....		44.3	
Ribonuclease, Sample A.....	300	12.3	72.2
“ “ B.....	300	40.0	9.7
“ “ C.....	300	37.5	15.3
Chymotrypsin.....	3	19.4	56.2
None.....		40.2	
Ribonuclease, Sample A.....	400	9.9	75.4
“ “ B.....	400	38.0	5.5
Chymotrypsin.....	4	12.1	69.9

contained an impurity not present in the others. In regard to the ability to remove PNA from liver mitochondria all three samples behaved alike. Thus the purity of crystalline ribonuclease preparations could not be judged on the basis of their ribonuclease activity.

*Effect of Chymotrypsin*—Cohen (7) reported that crystalline ribonuclease possessed proteolytic activity but was unable to decide whether the activity was due to an impurity or to an integral property of the enzyme. If the mechanism of the inhibition of the succinoxidase system involves proteolysis, it would seem that the proteolytic activity of ribonuclease preparations could be caused by impurities, since the three ribonuclease samples gave markedly different inhibitions of succinoxidase activity. Evidence that the mechanism of inhibition may be proteolytic and could be due to impurities is provided by experiments with chymotrypsin. This enzyme was found to inhibit the succinoxidase system and in much lower concentrations than were required in the case of ribonuclease (Table II). Trypsin was also found to inhibit succinoxidase activity but the concentrations required for inhibition were intermediate between the amounts of chymotrypsin and of ribonuclease needed for comparable inhibitions.

#### DISCUSSION

Zittle found that the inhibition of succinic dehydrogenase by ribonuclease could not be explained by the liberation of mononucleotides from tissue nucleoproteins, since the amounts of nucleotides released were insufficient to account for the inhibition (15). Furthermore, nucleotides were found to inhibit succinic dehydrogenase but not cytochrome oxidase (14, 15), whereas ribonuclease inhibited both enzymes (10). Our own results on the amounts of nucleic acid liberated by ribonuclease (presumably in the form of mononucleotides) support the conclusion of Zittle (15). In addition, our results indicate that the inhibition of succinic dehydrogenase by ribonuclease is apparently due to an impurity in the latter,<sup>1</sup> since different preparations of ribonuclease showed markedly different inhibitions although all samples had the same ribonuclease activity. It would appear that the ability to inhibit the succinoxidase activity may provide a sensitive tool for testing the purity of ribonuclease samples after they have been purified to the point of maximum ribonuclease activity.

The liberation of PNA from mitochondria by ribonuclease suggests that the position of the nucleic acid in the nucleoprotein structure may be terminal. Loring observed that ribonuclease did not attack the nucleic acid of tobacco mosaic virus and concluded that "nucleic acid would appear to occupy an integral rather than a terminal position in the virus particle, for in the latter case hydrolysis by ribonuclease might be expected" (9).

Cytologists have observed that ribonuclease destroys the basophilic properties of the cytoplasm (2, 3) and have considered this as evidence of the presence of PNA. The latter would not necessarily be the case, as Cohen has emphasized (7), if the result observed were due to the proteolytic properties of ribonuclease. On the other hand, it is not clear whether ribonuclease was acting on nucleic acid or nucleoprotein in the cytological work. It would seem possible that the fixation process might lead to destruction of the bond between nucleic acid and protein. The results of Zittle (15) and our own experiments on the liberation of PNA from mitochondria would appear to support the cytological observations. Extension of our experiments has shown that the action of ribonuclease is not confined to the PNA of liver mitochondria but that it will remove the major portion of the PNA from a variety of tissues.

The incomplete liberation of PNA observed in the present study may be analogous to the observation of Kunitz on the incomplete hydrolysis of yeast nucleic acid by ribonuclease (8). The results are probably not comparable, however, because in the former case the substrate was a nucleoprotein, while in the latter the substrate was a nucleic acid. Experiments are in progress to determine whether the PNA is released in the form of a polynucleotide or as mononucleotides. If the latter were the case, our results would appear to be comparable with those of Kunitz.

The question of whether the ribonucleoproteins of the cytoplasm possess any catalytic activity other than their assumed powers of protein synthesis must remain open for the present, and the significance of the association of succinoxidase activity with particles which include ribonucleoproteins must also remain undetermined.

#### SUMMARY

1. Crystalline ribonuclease was found to liberate 85 to 90 per cent of the pentose nucleic acid from the nucleoprotein of isolated rat liver mitochondria, without causing appreciable loss in succinoxidase activity. Prolonging the incubation time did not cause further amounts of nucleic acid to split off, but did cause decreases in succinoxidase activity to varying degrees, depending on the sample of ribonuclease. Thus, the presence of pentose nucleic acid was apparently unrelated to the activity of the succinoxidase system.

2. The inhibition of succinoxidase by crystalline ribonuclease previously observed was confirmed, but seemed to be due to an impurity, since other crystalline preparations of ribonuclease gave only slight inhibition.

3. The impurity may have been proteolytic in nature, since the succinoxidase system was inhibited by crystalline chymotrypsin present in a

concentration 0.01 as great as was required for comparable inhibition by ribonuclease.

4. The experiments remove some of the basis for any hypothesis that the succinoxidase system is a ribonucleoprotein or requires pentose nucleic acid for its activity.

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# THE EFFECTS OF THE VOLATILE ALDEHYDES FORMED ON THE ACCURACY OF THE MANOMETRIC NINHYDRIN-CARBON DIOXIDE METHOD IN ANALYSIS OF CERTAIN $\alpha$ -AMINO ACIDS

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In the reaction of primary  $\alpha$ -amino acids with ninhydrin, Ruhemann (1) showed that aldehydes,  $\text{CO}_2$ , and ammonia are formed:  $\text{RCH}(\text{NH}_2)\text{COOH} \rightarrow \text{RCHO} + \text{CO}_2 + \text{NH}_3$ . Van Slyke, Dillon, MacFadyen, and Hamilton (2) developed manometric and titrimetric determinations of the  $\text{CO}_2$  as specific methods for free amino acids, and showed that only free amino acids, but not peptides, evolve  $\text{CO}_2$  from their carboxyl groups.

In analyses of valine and the leucine isomers by the manometric procedure, Schott, Rockland, and Dunn (3) have found it advantageous to add hydrazine to the alkali used for absorbing the  $\text{CO}_2$  in the Van Slyke-Neill gas chamber, in order to bind the aldehydes formed from these amino acids; otherwise they found that these aldehydes were sufficiently volatile to accompany the  $\text{CO}_2$  into the final gas phase and cause plus errors of several per cent in the  $\text{CO}_2$  estimation. The  $\text{NaOH-N}_2\text{H}_4$  reagent used was that employed by Van Slyke and Folch (4) to prevent error from halogens in manometric carbon combustions.

The technique used by Schott, Rockland, and Dunn differed from that used for the ninhydrin- $\text{CO}_2$  method in this laboratory by minor variations which it appeared might magnify the effect of the aldehydes. It appeared desirable therefore to check the results with valine and the leucines, and also to ascertain whether the aldehydes cause measurable errors in determinations of the free amino acids in biological mixtures, such as protein hydrolysates and blood plasma, when reagents without hydrazine are employed.

## EXPERIMENTAL

**Apparatus**—The Van Slyke-Neill manometric apparatus (5), water bath, calibrated glass spoons, and alkali storage vessels were those previously described (2). All-glass reaction vessels with high temperature stop-cock grease described by Hamilton and Van Slyke (6) were employed throughout.<sup>1</sup>

<sup>1</sup> Schott, Rockland, and Dunn (3) apparently used the all-glass reaction vessel only for methionine, the analysis of which they found exact.

*Reagents*—Ninhydrin, solid citrate buffers, 2.0 N lactic acid, 5 N sodium hydroxide, and nearly carbon dioxide-free 0.5 N sodium hydroxide were prepared according to Van Slyke, Dillon, MacFadyen, and Hamilton (2), and approximately carbon dioxide-free 0.5 N sodium hydroxide and 2.0 N lactic acid in 25 per cent sodium chloride solution as described by MacFadyen (7).<sup>2</sup> The "0.5 N sodium hydroxide and 0.15 M hydrazine" in 25 per cent sodium chloride solution was prepared as by Schott, Rockland, and Dunn (3); enough 18 N CO<sub>2</sub>-free NaOH was added to 25 per cent NaCl to make the alkali 0.5 N, and hydrazine sulfate crystals were added to make the hydrazine concentration 0.15 M.

It was found that, for eliminating the aldehyde error, the hydrazine is equally efficient when it is added to the 2 N lactic acid-sodium chloride solution. Over the alkaline hydrazine solution the acid solution has the advantage that in the acid the hydrazine is stable, whereas it slowly decomposes in alkaline solution (4). 2 gm. of hydrazine sulfate are dissolved in 100 cc. of the 2 N lactic acid-NaCl solution (this is about all the hydrazine that the solution will dissolve). Though the concentration of hydrazine in the chamber after neutralization of the alkali is approximately 0.05 M (in the technique of Schott *et al.* the concentration of hydrazine in the chamber after neutralization is approximately 0.10 M) it is apparently sufficient to combine with all the volatile aldehyde in the chamber, for analytical results are identical, whether the hydrazine is included in the alkali or in the acid reagents.

*Amino Acids*<sup>3</sup>—Determinations for carbon, nitrogen (Dumas), hydrogen, moisture, ash, and amino nitrogen by the nitrous acid method (9) were carried out on all amino acids to assess their purity before analysis by the ninhydrin-carbon dioxide method. All values reported are corrected for moisture and ash content. Results of these analyses are given in Table I.

*Experimental Procedures*—For the ninhydrin-CO<sub>2</sub> determinations a sample of about 100 mg. of each amino acid was transferred to a 25 cc.

<sup>2</sup> The use of NaCl-saturated reagents in the gas chamber has an advantage in providing a final acidified solution in which CO<sub>2</sub> is but slightly soluble. Such reagents are of particular advantage in microanalyses, in which the volume of extracted CO<sub>2</sub> gas is reduced to 0.5 cc. before the pressure is measured. When reagents without the NaCl are used, an average of 3.7 per cent of the CO<sub>2</sub> is reabsorbed during the interval when the gas volume is being diminished from nearly 50 cc. to the final 0.5 cc. for the manometric pressure reading. This reabsorption is corrected for by the *i* factor of 1.037 of Van Slyke and Neill (5, 8), but variations in technique may cause variations of several units in the last figure of the factor. When the salt-saturated reagents are used, the *i* factor is reduced to 1.006, and variations in it to  $\pm 0.001$ . The salt-saturated reagents were introduced by MacFadyen (7) for microdeterminations of carboxyl nitrogen in plasma, and were adopted by the writers (6) for similar analyses. MacFadyen showed that use of the salt-saturated reagents caused no significant errors in determinations of carboxyl nitrogen of arginine, aspartic acid, or the mixture of amino acids in hydrolyzed gelatin.

<sup>3</sup> The authors are indebted to Dr. A. Elek of The Rockefeller Institute for Medical Research for the elementary analyses reported in this paper.

volumetric flask, dissolved, and made to volume with distilled water. A sample of 1 cc., containing approximately 4 mg. of amino acid, was used for each analysis. All solutions were analyzed on the day prepared. The reaction of each amino acid solution with ninhydrin was carried out at pH 2.5 as described by Van Slyke, Dillon, MacFadyen, and Hamilton (2).

With each amino acid solution, parallel ninhydrin-CO<sub>2</sub> analyses were carried out in which three different types of solutions were used in the Van Slyke-Neill chamber: (a) The CO<sub>2</sub> was absorbed, as in the original general method (2) by 2 cc. of 0.5 N NaOH solution, and then set free by 1 cc. of 2 N lactic acid; (b) the same as (a) except that the 0.5 N NaOH and 2 N lactic acid were prepared in a salt solution made by dissolving 250 gm. of NaCl in 750 cc. of water (7); (c) the same as (b), except that the salt-

TABLE I  
*Analytical Data for Amino Acids Investigated*

Amino acid	Source	Moisture	Ash	Analysis of dry substance (corrected for ash content) as per cent of calculated theoretical			
				Carbon	Hydrogen	Nitrogen (Dumas)	Amino nitrogen (Van Slyke (9))
		per cent	per cent	per cent	per cent	per cent	per cent
dl-Alanine.....	Eimer and Amend	0.07	0.07	99.6	101.0	99.8	99.4
dl-Valine.....	Merck	0.47	0.03	100.0	100.5	99.8	100.0
l-Leucine.....	"	0.58	0.07	99.8	99.8	100.4	100.0
dl-Isoleucine.....	"	0.10	0.23	99.6	100.2	98.8	99.5
dl-Norleucine.....	"	0.27	0.10	100.0	100.3	99.1	99.5

saturated 0.5 N NaOH solution contained hydrazine. CO<sub>2</sub> pressures were measured with the gas at 2 cc. volume.

The previous finding from this laboratory (2) was confirmed, that six to-and-fro excursions of the gases between reaction vessel and Van Slyke-Neill chamber are sufficient to cause complete transfer of CO<sub>2</sub> from the vessel to the alkali solution in the chamber, when the volume of solution in the extraction vessel is 1 cc. and its temperature 38° at the beginning of the excursions. Previous findings also were confirmed that after the carbonate solution in the chamber is acidified, 3 minutes shaking *in vacuo* are sufficient to obtain maximal extraction of the CO<sub>2</sub> when the solutions are saturated with NaCl, 1.5 minutes when NaCl is not used. The extra labor and time entailed by the eleven excursions and 5 minutes shaking used by Schott, Rockland, and Dunn (3) are unnecessary.

The *c* corrections were established by blank analyses performed with all

the reagents, including the ninhydrin. As previously pointed out (6) some lots of ninhydrin contribute slightly, but measurably, to the *c* correction.

*Calculations*—Calculations of the carboxyl (or  $\alpha$ -amino) nitrogen from CO<sub>2</sub> pressures were made by the factors of Van Slyke *et al.* (2) when the 0.5 N sodium hydroxide and 2 N lactic acid used in the Van Slyke-Neill chamber were simple aqueous solutions. When the solutions of lactic acid and NaOH used in the gas chamber, with or without hydrazine, were prepared

TABLE II  
*Analysis of Amino Acid Solutions*

All analyses were carried out in duplicate or triplicate. Reaction with ninhydrin was at pH 2.5. The tabulated results indicate the percentages of theoretical carboxyl nitrogen values.

Amino acid analyzed	Present results			Results of Schott, Rockland, and Dunn (3)*		
	Original technique of Van Slyke <i>et al.</i> (2) No NaCl; no hydrazine (a)	Chamber reagents approximately saturated with NaCl (7)		Original technique of Van Slyke <i>et al.</i> (2) No NaCl; no hydrazine (d)	Chamber reagents approximately saturated with NaCl (7)	
		No hydrazine (b)	Hydrazine added (c)		No hydrazine (e)	Hydrazine added (f)
Alanine.....	100.1	100.2	99.5	100.4	102.2	100.2
Valine.....	102.6	106.5	100.1	104.4	112.2	100.1
Leucine.....	102.6	106.6	99.6	104.6	114.5	100.1
Isoleucine.....	103.6	107.0	99.6	105.4	113.7	99.9
Norleucine.....	100.5	103.2	99.9	101.2	104.1	100.0

\* In Tables I, II, and III of Schott *et al.*, the column heading "Original technique" is not quite exact; the technique used, as indicated elsewhere in the paper (3), was not the original technique of Van Slyke, Dillon, MacFadyen, and Hamilton (2) but was modified by use of salt-saturated reagents. Only in Table IV of Schott *et al.* were the results under the column heading "Original technique" apparently obtained by that technique.

in 25 per cent NaCl solution (see Table II) the factors of MacFadyen (7) were used.

### Results

*Analyses of Individual Amino Acids*—When analyzed by the original technique of Van Slyke, Dillon, MacFadyen, and Hamilton (2) (column (a), Table II) results higher than theoretical were obtained with valine and the leucines as reported by Schott *et al.* (column (d), Table II), but the plus errors were only about one-half those reported by Schott *et al.* When NaCl-saturated solutions were used in the gas chamber, the plus errors were increased, but again were only about one-half as great as reported by Schott *et al.* (column (e), Table II).

The experiments of Table III were performed in an unsuccessful attempt to find the reason for our failure to obtain in the analyses without hydrazine plus errors as large as those reported by Schott, Rockland, and Dunn. From the second column it is apparent that, in transferring the  $\text{CO}_2$  from the reaction vessel to the alkali in the chamber, increasing the number of excursions from six to eleven makes no difference, nor does increasing from 3 minutes to 5 the interval during which the solution in the gas chamber is

TABLE III  
*Carboxyl Nitrogen Determinations on Valine*

The results are expressed as percentages of the theoretical carboxyl nitrogen. All analyses were carried out in duplicate or triplicate with reactions at pH 2.5.

Conditions for transfer of $\text{CO}_2$ to gas chamber and for extraction of $\text{CO}_2$ from acidified solution in chamber*	Ninhydrin reaction in solution of 1 cc. volume			Ninhydrin reaction in solution of 5 cc. volume		
	Original technique of Van Slyke <i>et al.</i> (2) No NaCl, no hydrazine	Chamber reagents approximately saturated with NaCl (7)		Original technique of Van Slyke <i>et al.</i> (2) No NaCl; no hydrazine	Chamber reagents approximately saturated with NaCl (7)	
		No hydrazine	Hydrazine added		No hydrazine	Hydrazine added
6 excursions, 38°, 3 min. shaking	101.2	106.4	99.8	101.7	106.0	99.4
11 excursions, 38°, 5 min. shaking		106.6	99.6		106.6	
6 excursions, 25°, 3 min. shaking		105.0				
6 excursions, 38°, 3 min. shaking		106.6				
6 excursions, 45°, 3 min. shaking		108.0				

\* "Excursions" were to transfer  $\text{CO}_2$  from the reaction vessel to the alkali solution in the gas chamber. See (2) p. 645. The temperature figures indicate the temperature of the solution in the reaction vessel when the transfer was started. "Minutes shaking" are to extract the  $\text{CO}_2$  from solution after acidification of the latter in the chamber. See (4) p. 525.

shaken after acidification to extract the  $\text{CO}_2$  for measurement. In all of the analyses of Schott *et al.* the reaction between amino acid and ninhydrin was carried out in a solution of only 1 cc. volume in the reaction chamber, whereas the writers usually use 2 cc., and in analyses of blood filtrates 5 cc. It was thought that more aldehyde might escape from the smaller volume of solution, during transfer of the  $\text{CO}_2$  gas to the alkali in the chamber, and consequently cause greater plus error. However, the results in the last three columns of Table III indicate that the error was no less when the ninhydrin reaction was carried out in 5 cc. of solution.

the reagents, including the ninhydrin. As previously pointed out (6) some lots of ninhydrin contribute slightly, but measurably, to the *c* correction.

*Calculations*—Calculations of the carboxyl (or  $\alpha$ -amino) nitrogen from CO<sub>2</sub> pressures were made by the factors of Van Slyke *et al.* (2) when the 0.5 *N* sodium hydroxide and 2 *N* lactic acid used in the Van Slyke-Neill chamber were simple aqueous solutions. When the solutions of lactic acid and NaOH used in the gas chamber, with or without hydrazine, were prepared

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in 25 per cent NaCl solution (see Table II) the factors of MacFadyen (7) were used.

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The experiments of Table III were performed in an unsuccessful attempt to find the reason for our failure to obtain in the analyses without hydrazine plus errors as large as those reported by Schott, Rockland, and Dunn. From the second column it is apparent that, in transferring the  $\text{CO}_2$  from the reaction vessel to the alkali in the chamber, increasing the number of excursions from six to eleven makes no difference, nor does increasing from 3 minutes to 5 the interval during which the solution in the gas chamber is

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		No hydrazine	Hydrazine added		No hydrazine	Hydrazine added
6 excursions, 38°, 3 min. shaking	101.2	106.4	99.8	101.7	106.0	99.4
11 excursions, 38°, 5 min. shaking		106.6	99.6		106.6	
6 excursions, 25°, 3 min. shaking		105.0				
6 excursions, 38°, 3 min. shaking		106.6				
6 excursions, 45°, 3 min. shaking		108.0				

\* "Excursions" were to transfer  $\text{CO}_2$  from the reaction vessel to the alkali solution in the gas chamber. See (2) p. 645. The temperature figures indicate the temperature of the solution in the reaction vessel when the transfer was started. "Minutes shaking" are to extract the  $\text{CO}_2$  from solution after acidification of the latter in the chamber. See (4) p. 525.

shaken after acidification to extract the  $\text{CO}_2$  for measurement. In all of the analyses of Schott *et al.* the reaction between amino acid and ninhydrin was carried out in a solution of only 1 cc. volume in the reaction chamber, whereas the writers usually use 2 cc., and in analyses of blood filtrates 5 cc. It was thought that more aldehyde might escape from the smaller volume of solution, during transfer of the  $\text{CO}_2$  gas to the alkali in the chamber, and consequently cause greater plus error. However, the results in the last three columns of Table III indicate that the error was no less when the ninhydrin reaction was carried out in 5 cc. of solution.

We are at a loss to account for the fact that, although our results qualitatively confirm those of Schott *et al.*, to the effect that the aldehydes generated by valine and the leucines cause a plus error unless hydrazine is used, the error we find is only half as great as that reported by these authors.

*Analysis of Protein Hydrolysates*—Gelatin and edestin were hydrolyzed by refluxing with 20 cc. of 6 N hydrochloric acid per gm. of protein for 24 hours. Edestin was chosen because protein analyses in the literature

TABLE IV

*Analysis of Acid Protein Hydrolysates by Ninhydrin-Carbon Dioxide Method*

Protein acid hydrolysate	Carboxyl nitrogen* in sample analyzed. Reagents in gas chamber in 25 per cent NaCl solution	
	No hydrazine	With hydrazine
	mg.	mg.
Gelatin.....	0.1551	0.1557
Edestin.....	0.289	0.286

\* "Carboxyl nitrogen" is used, as in previous papers (2, 6), to indicate nitrogen calculated as 1 atom of N per molecule of CO<sub>2</sub> evolved by reaction with ninhydrin.

TABLE V

*Analysis of Picric Acid Filtrate of Dog and Human Plasma for Carboxyl Nitrogen by Method of Hamilton and Van Slyke (6)*

Plasma	Carboxyl nitrogen content*	
	Analyses without hydrazine	Analyses with hydrazine
	mg. per 100 cc.	mg. per 100 cc.
Dog 1.....	4.39	4.44
" 2.....	4.00	4.04
" 3.....	4.07	4.14
Human 1.....	4.09	4.00
" 2.....	4.26	4.32
" 3.....	4.94	5.02

\* All analyses reported are the averages of duplicate determinations agreeing to within 1 per cent.

indicate that edestin is especially high in content of the leucine-valine group. 3 cc. of gelatin hydrolysate or 5 cc. of edestin hydrolysate were placed in a 100 cc. volumetric flask, neutralized with NaOH, and diluted to 100 cc. with distilled water. 1 cc. samples were used for the ninhydrin-CO<sub>2</sub> determination, the reaction with ninhydrin being at pH 2.5. The 0.5 N NaOH and 2.0 N lactic acid used in the gas chamber were prepared in 25 per cent NaCl solution, hydrazine being either added or omitted in the NaOH solution.



The results are given in Table III. Addition of hydrazine to the salt-saturated reagents lowered the carboxyl nitrogen yield by 1.0 per cent in the case of edestin and by 0.4 per cent in the case of gelatin (Table IV), the effect being about what would be estimated from the reported (10) content of valine and the leucines in the respective proteins; *viz.*, 7 per cent in gelatin and 20 per cent in edestin.

*Analysis of Plasma Filtrates*—Analysis of picric acid filtrates of fresh dog and human plasma, with the salt-saturated reagents with and without added hydrazine, are given in Table V. The plasma carboxyl nitrogen values were not decreased by adding hydrazine; in fact their average with hydrazine was 0.03 mg. per 100 cc. higher than without hydrazine. It accordingly appears, even when salt-saturated reagents are employed, that the use of hydrazine is unnecessary in order to avoid plus errors in determination of plasma amino acids by the ninhydrin- $\text{CO}_2$  method.

#### DISCUSSION

Routine addition of 2 gm. of hydrazine sulfate per 100 cc. to the lactic acid used in the manometric method (2) appears to be desirable to prevent small plus errors from the volatile aldehydes yielded by valine and the leucines. It is true that, unless this group of amino acids forms 20 per cent or more of the total in the mixture analyzed, the effect of the aldehydes is negligible, as in blood filtrates. The addition of the hydrazine, however, adds nothing to the manipulations of the analysis, and it increases precision when the leucine-valine group forms an important fraction of the total amino acids present.

#### SUMMARY

Schott, Rockland, and Dunn (3) are confirmed, that, in determinations of carboxyl nitrogen of valine and the leucines by the manometric ninhydrin- $\text{CO}_2$  method of Van Slyke, Dillon, MacFadyen, and Hamilton (2), addition of hydrazine to the reagents in the gas chamber increases the accuracy by abolishing a plus error (of 0.5 to 3.6 per cent according to present results) caused by volatile aldehydes formed from these amino acids.

Analyses of other amino acids are not affected by the aldehydes formed, and in determinations of free amino acids in biological mixtures, such as protein hydrolysates and blood filtrates, use of hydrazine lowers the results by less than 1 per cent. Nevertheless hydrazine adds a factor of safety to the method, and the addition of 2 gm. of hydrazine sulfate per 100 cc. to the 2 N lactic acid used as reagent in the manometric method is recommended as a routine procedure.

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## OXYGEN POISONING

### X. THE EFFECT OF OXYGEN AT EIGHT ATMOSPHERES UPON THE OXYGEN CONSUMPTION OF THE INTACT MOUSE\*

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(Received for publication, March 8, 1946)

In a series of papers on the subject of oxygen poisoning (1), we have reported experiments on the effect of oxygen at 8 atmospheres upon the activity of enzymes in the presence of specific substrates and the oxygen uptake of surviving tissues of the rat. In this paper we report further experiments in which the oxygen uptake of the intact mouse was measured at 8 atmospheres of oxygen.

#### *Methods*

*Respirometer*—This was a glass cylinder 4 cm. in diameter and 15 cm. long. One end was contracted into a tube of about 3 mm. in diameter fitted with a stop-cock. The other end was closed by a ground glass cover (4 cm. diameter) which in turn was also extended into a 3 mm. tube without a stop-cock. Each end of the cylinder contained a small wire gauze canister containing Dioxorb for the absorption of  $\text{CO}_2$ . The space between the two canisters was just about large enough to contain a mouse.

Following assembly, the respirometer was immersed in a water bath at room temperature. The tube at one end was connected with a source of 100 per cent oxygen. The other end was connected to a mercury manometer. The manometer was equipped with a 2-way stop-cock permitting the respiratory chamber to be connected at will with either the manometer arm or a supply of 100 per cent oxygen. The respirometer could be used either at 1 atmosphere or, in a pressure chamber, at 8 atmospheres.

*Operation*—The respirometer was first flushed with 100 per cent oxygen and then closed except that by adjustment of the manometer stop-cock the respirometer chamber was connected with the manometer arm. The change of mercury levels in the manometer was then measured. When the capacity of the manometer was reached, the stop-cock was turned to admit fresh oxygen either at 1 or 8 atmospheres and new readings of the manometer levels were begun immediately. This cycle was repeated during the

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

course of the observations. Except for the few seconds required to admit new oxygen, a continuous record of oxygen uptake was thus obtained.

Micromoles of oxygen uptake were calculated from the manometer readings as follows: The volume of the respirometer was measured. The weight of the Dioxorb and mouse was known, and their volume was calculated, assuming a density of 1. The difference of these two volumes gave the free space in the respirometer. The appropriate factor converting changes of mercury level to micromoles of oxygen consumption could then be easily calculated.

*Efficiency of CO<sub>2</sub> Absorption*—In any respiring system containing an alkali inset to absorb CO<sub>2</sub>, the rate of absorption of CO<sub>2</sub> will be a function of its partial pressure. Initially the CO<sub>2</sub> pressure will increase until the rate of absorption is equal to the rate of production. Thereafter no change of CO<sub>2</sub> occurs and change in the volume of the gas in the respirometer will be the true oxygen consumption. Careful preliminary control experiments showed that the "steady state  $P_{CO_2}$ ," in our respirometer was approximately 40 to 45 mm. of Hg and was reached in 9 to 10 minutes from the time of flushing the apparatus with oxygen. In all of our experiments, the time of assembly of the apparatus containing the mouse was 20 to 45 minutes. Hence the "steady state" was established before any of the recorded observations were made. Since our experiments are entirely of a comparative nature, we do not believe that the validity of any of our conclusions is impaired by the presence of this relatively high CO<sub>2</sub> during the period of determination of oxygen uptake.

*Residual CO<sub>2</sub> Absorption*—When oxygen consumption ceased by reason of the death of the mouse within the chamber, the manometer level continued to fall. This is due to absorption of the "steady state" CO<sub>2</sub> which during the period of oxygen consumption is maintained at a constant level of approximately 40 mm. of Hg. This explains why the graphs in Fig. 3 continue to show an apparent oxygen uptake after the animal has died. This artifact does not invalidate any of the conclusions made in this paper because residual CO<sub>2</sub> absorption is easily detectable within 1 to 2 minutes following cessation of oxygen uptake.

*Physical Solution of O<sub>2</sub>*—In any system in which gas changes are measured immediately after increasing the pressure, misinterpretation due to physical solution of the gas in water or other substances must be guarded against. Dioxorb absorbs considerable amounts of oxygen when the pressure is elevated. By reducing the amount of Dioxorb and elevating the pressure to 8 atmospheres in two stages, errors from this source were made insignificant.

Further validation of the method used here comes from the fact that our measurements of basal oxygen uptake of the mouse are close to stand-

ard values quoted in the literature (2 micromoles per gm. of body weight per minute).

#### EXPERIMENTAL

*Oxygen Uptake of Mouse at 1 Atmosphere of Oxygen and 7 Atmospheres of Nitrogen*—As an added control of the method and to test the effect of excess pressure of an indifferent gas upon the oxygen uptake, an experiment was done under 1 atmosphere of oxygen and 7 atmospheres of nitrogen. A 24 gm. mouse was anesthetized with an intraperitoneal injection

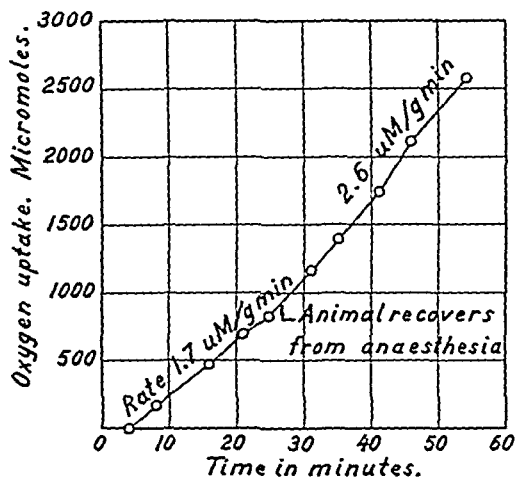


FIG. 1. The oxygen uptake of a mouse under 1 atmosphere of oxygen and 7 atmospheres of nitrogen.

of 1.2 mg. of nembutal. About 26 minutes were required to assemble the apparatus and elevate the pressure. Fig. 1 shows the subsequent course of the oxygen uptake. For the first 25 minutes, during which the animal was quiet, the oxygen uptake was 1.7 micromoles per gm. per minute, close to the standard basal value. Thereafter the anesthesia wore off and the mouse became active. The oxygen uptake increased and continued at a uniform rate of 2.6 micromoles per gm. per minute. The increased pressure of nitrogen had no measurable effect upon the oxygen uptake.

*Oxygen Uptake at 8 Atmospheres of Oxygen*—Several control experiments were done in which the oxygen uptake of mice was measured first at 1 atmosphere and then at 8 atmospheres. In no case was a measurable difference observed between the two rates.

Complete data of a similar experiment are shown in Fig. 2. In this case the animal was unanesthetized. The readings were begun 2 minutes

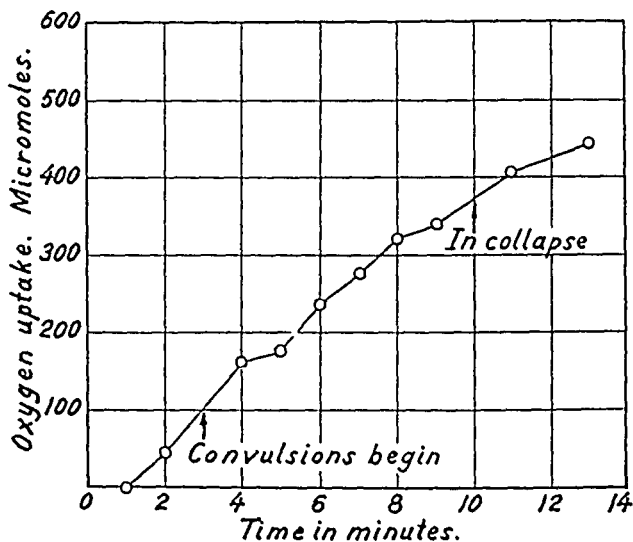


FIG. 2. The oxygen uptake of a mouse under 8 atmospheres of oxygen

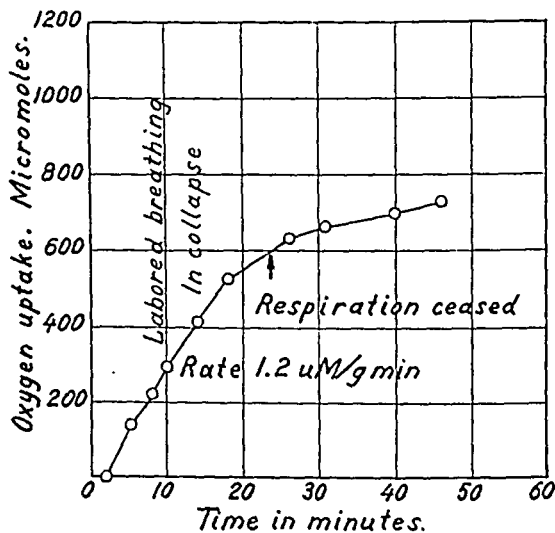


FIG. 3. The oxygen uptake of an anesthetized mouse under 8 atmospheres of oxygen.

after the oxygen pressure reached 8 atmospheres. The animal was active and the initial rate of oxygen uptake was 2.5 micromoles per gm. per minute. Toxic symptoms developed rapidly. Within 2 minutes, a generalized

convulsion occurred. Further convulsions recurred intermittently. At 10 minutes the animal was in collapse, with severe gasping respirations. The exact time of death could not be determined, but occurred 5 to 10 minutes after the observations on the oxygen uptake were discontinued. No significant decrease in the rate of oxygen uptake was observed during the first 10 to 12 minutes of exposure when the animal had severe manifestations of oxygen poisoning.

A longer period of observation of oxygen uptake is recorded in Fig. 3. In this case the mouse was anesthetized with nembutal. Evidences of oxygen toxicity following elevation of the pressure to 8 atmospheres quickly developed. At about 8 minutes the characteristic labored breathing developed and at about 12 to 13 minutes the animal was in a state of collapse. Respirations ceased at approximately 23 minutes. Owing to the anesthetic no convulsions occurred at any time. *Despite these patent evidences of a severe toxic state, there was no measurable change in the oxygen uptake.* At 15 to 18 minutes the oxygen uptake began to fall off, and the animal died at approximately 22 minutes.

The residual  $\text{CO}_2$  absorption discussed under "Methods" is observed in this experiment.

#### DISCUSSION

Since the time of Bert the train of deleterious symptoms which develops with greater or less rapidity in animals exposed to high pressures of oxygen has been described by the collective term "oxygen poisoning." For example, when rats (2) are exposed to 8 atmospheres of oxygen, tonic and clonic spasms begin in 2 to 4 minutes, followed by severe generalized convulsions, labored breathing increasing in severity with time until within 15 to 20 minutes the animal is in antemortem collapse. Death occurs usually within 20 to 30 minutes. In man, facial twitching, muscular incoordination, and mental disorientation have been observed within 2 to 3 minutes at 3 atmospheres of oxygen. Analogous symptoms have been observed in many species of animals exposed to oxygen at 1 or more atmospheres for varying times. Despite a considerable amount of experimentation, the mechanism by which oxygen at high pressures causes these changes in normal function is unknown. Bert suggested that cellular enzymatic systems, particularly those concerned with oxygen utilization, are inactivated. In effect there would be a tissue anoxemia of sufficient degree to cause profound metabolic disturbance responsible for the symptoms. The condition has been likened to cyanide poisoning and the responses to low oxygen, but of sufficiently unusual character to warrant a special appellation, *hyperoxic anoxia*. This hypothesis has been widely discussed

in the literature and some investigators have concluded that hyperoxic anoxia is the main, if not the sole, cause of the toxic action of oxygen.

In a series of studies (2) on the effects of oxygen at high pressure on the metabolism of tissue slices or homogenates and enzyme preparations, we have been unable to find any evidence to support this hypothesis. To be sure, we have observed that high oxygen inactivates enzymes as indicated by either a decrease of their action upon specific substrates or a decrease of the total oxygen uptake of tissue slices. But we have always been insistent that in evaluating these results a careful consideration must be given to the time factors involved. With no exceptions, we have found that the consumption of oxygen and the production of  $\text{CO}_2$  *in vitro* at 8 atmospheres of oxygen by *intact tissue slices* were not significantly different from values observed at 1 atmosphere during the initial period of 30 to 60 minutes. Thereafter there is a slowly progressing decrease which, at the end of 120 minutes, ranges from 60 to 80 per cent. We have also studied the effect of high oxygen on many enzymes of major metabolic importance. Among these may be mentioned succinic, malic, and glutamic dehydrogenases, and  $\alpha$ -ketoglutarate and pyruvate oxidases. With these, the situation is quite similar to that found in the intact mouse: there is essentially no inactivation for the initial period of 20 to 40 minutes; thereafter it progresses slowly. In contrast, many enzymes are not affected at all by exposure to 8 atmospheres of oxygen for  $\frac{1}{2}$  hours; *e.g.*, cytochrome oxidase, lactic dehydrogenase, uricase, catalase, carbonic anhydrase, cholinesterase. Xanthine oxidase, choline acetylase, and *D*-amino acid oxidase in homogenized tissues or extracts have been found to be rapidly inactivated by high oxygen pressure. However, the two latter are completely unaffected when their activity is measured in intact tissue. Furthermore, various tissues (as well as enzyme preparations) from animals which have been killed by exposure to 8 atmospheres of oxygen have consistently shown normal metabolic activities as indicated by oxygen uptake. In the case of dogs poisoned by prolonged exposure to 1 atmosphere of oxygen, we found no significant change in the oxygen uptake of brain or lung slices.

On the basis of these findings, we have characterized enzymatic inhibition by high oxygen pressure as *subacute*. That is to say, it requires a relatively long time to develop.

In sharp contrast to the slow onset of the subacute phase is the rapid development of symptoms observed in the intact animal under high oxygen pressure. The characteristics of this *acute phase* have already been described. From our experiments already reported it was difficult to see how the acute phase could be attributed to a rapid inactivation of enzymes with resulting significant decrease of oxygen uptake by the tissues. The



experiments reported in this paper constitute a strong corroboration of this opinion. For we have shown that mice at 8 atmospheres of oxygen develop with great rapidity the severest type of symptoms and may be brought practically to the point of death *without showing any measurable decrease in the total oxygen uptake*. We are, therefore, forced to conclude that the probability is very small that hyperoxic anoxia, as it has been defined here, is the cause of the acute phase of oxygen poisoning. A reservation is necessary because the oxygen consumption of the brain is small relative to the total. Hence, the complete cessation of oxidative processes in the central nervous system might remain undetected in our experiments if the extraneural metabolism was unaffected. However, our experiments have abundantly shown that brain tissue is no exception to the general rule that the oxidative processes are but slowly affected by high oxygen pressure. Hence we feel that little weight need be given to this reservation. On the other hand, we are not prepared to exclude the possibility that enzymatic inhibitions may play a rôle in the acute phase. For it is quite conceivable that some particular enzyme by virtue of its nature or topical situation in the cell is, unlike most enzymes, *rapidly inactivated* by high oxygen pressure. If this enzyme is peculiarly essential for normal functioning of the nervous or other tissue, while playing a relatively small rôle in the total metabolism, its inactivation might explain why, without significant changes in *total oxygen consumption*, normal function is impaired or lost. Among such special enzymatic activities we have studied acetylcholine synthesis by brain, phosphorylation of glucose by kidney, and carbonic anhydrase and catalase activity of blood and brain. We have found no indications whatever that inactivation of these enzymes plays any rôle in the acute phase of oxygen poisoning.

The further possibility that a toxic substance is formed in the tissues by high oxygen pressure must certainly be considered, but as yet there is little evidence to support it.

## SUMMARY

1. Intact mice under 8 atmospheres of oxygen rapidly developed severe symptoms of oxygen poisoning without showing measurable decreases of oxygen consumption.
2. On the basis of this and previous observations, it is concluded that hyperoxic anoxia is not the cause of the acute phase of oxygen poisoning.

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## OXYGEN POISONING

### XI. THE RELATION BETWEEN INACTIVATION OF ENZYMES BY OXYGEN AND ESSENTIAL SULFHYDRYL GROUPS\*

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The effect of oxygen at high pressure on a number of enzymatic reactions has been reported in previous papers of this series. The enzymes studied so far can be divided into two groups: (a) those more or less rapidly inactivated by oxygen and (b) those resistant to oxygen.

Stadie, Riggs, and Haugaard (1) reviewed the literature and discussed possible chemical mechanisms involved in the inactivation of enzymes by oxygen.

One of these mechanisms is of particular interest; namely, inactivation of the so called sulfhydryl enzymes by oxidation of essential sulfhydryl groups.

The sulfhydryl enzymes are in general inactivated by oxidizing agents such as porphyrindin, iodine, iodosobenzoate, cystine, and oxidized glutathione. That atmospheric oxygen alone under certain conditions inactivates sulfhydryl enzymes has been found in several cases. Some sulfhydryl enzymes, while stable in the presence of air, are inactivated by oxygen at higher pressure. As was first shown by Lehman (2), succinic dehydrogenase is an example. This enzyme, in tissue homogenates and slices, is completely inactivated by 2 to 4 hours exposure to oxygen at 7 atmospheres (3).

A problem of obvious interest, therefore, is to determine whether or not there is a correlation between inactivation of enzymes by oxygen and the presence of essential sulfhydryl groups. To study this question, the effect of oxygen on a number of enzymes, both with and without essential sulfhydryl groups, has been investigated.

Experiments on the effect of oxygen at high pressure on phosphorylation of glucose in kidney extract are also reported. These experiments were done in order to determine whether a phosphorylating mechanism in tissue is more susceptible to the inactivating action of oxygen than oxida-

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

tive reactions. In general, we have found oxidative reactions to be only slowly affected by oxygen at high pressure.

### Methods

A procedure similar to the one adopted in previous studies has been used here. Tissue slices, homogenates, or extracts were exposed to oxygen at high pressure in a preliminary period and subsequently assayed for the activity of the particular enzyme or enzyme system studied. Part of the enzyme preparations were exposed to nitrogen, air, or oxygen as controls.

The following were studied: lactic, malic, *l*(+)-glutamic, and choline dehydrogenases, pyruvic and  $\alpha$ -ketoglutaric oxidases, acid phosphatase, and the system phosphorylating glucose in kidney extract.

*Lactic Dehydrogenase*—Three different preparations were used: viz., rat liver homogenate, pigeon breast muscle extract, and cells of *Escherichia coli* disintegrated by sonic vibration. In the first two tissues, the activity was determined by the ferricyanide technique originally described by Quastel and Wheatley (4). The carbon dioxide output was measured for about 30 minutes at 38° and the enzymatic activity calculated after correction for the carbon dioxide evolved in a sample without added substrate. The liver homogenate was active without the addition of diphosphopyridine nucleotide, in contrast to pigeon breast muscle extract which required the addition of 2 mg. of a crude diphosphopyridine nucleotide preparation for full activity.

The lactic dehydrogenase from *Escherichia coli* was prepared by washing cells grown in a lactate-containing medium, resuspending them in saline, and disintegrating them by sonic vibration for 1 hour at room temperature. The activity was determined by measuring the oxygen uptake in a Warburg vessel at 38° in the presence of methylene blue, phosphate buffer, and lactate. The preparations had no oxygen uptake in the absence of substrate.

*Malic Dehydrogenase*—Rat liver and kidney homogenates were used and the activity determined by the ferricyanide technique. It was necessary to add cyanide (0.020 M) which reacts with the oxalacetic acid formed, thus removing it from the reaction. About 1 mg. of a crude preparation of diphosphopyridine nucleotide was also added.

*Choline and Glutamic Dehydrogenases*—Activity of these enzymes was determined in rat liver and kidney slices by the ferricyanide technique. The components of the medium were the same as in the case of lactic dehydrogenase. The rate of carbon dioxide evolution was determined in the presence and absence of choline or *l*(+)-glutamate and the activity per gm. of wet tissue was calculated after correction for the carbon dioxide evolved in the absence of substrate.

*Pyruvic Oxidase*—Rat brain homogenate was prepared in a phosphate-saline medium of pH 7.2 containing 0.002 M  $MgCl_2$  and 0.001 M  $MnCl_2$ . Utilization of pyruvate during 1 hour's equilibration in air at 38° was determined.

*$\alpha$ -Ketoglutaric Oxidase*—The oxidation of  $\alpha$ -ketoglutarate by rat kidney slices in the presence of malonate was determined as described by Barron and Singer (5).

*Acid Phosphatase*—Acid phosphatase activity of rat kidney homogenate was determined as described by Shinowara, Jones, and Reinhart (6).

*Phosphorylation in Kidney Extract*—Phosphorylation of glucose in the presence of fluoride was determined in rabbit kidney extract according to the method of Colowick, Welch, and Cori (7).

### Results

In the case of lactic dehydrogenase in preparations from rat liver homogenates and pigeon breast muscle extracts, it was impossible to work at 38° because the preparations both in air and at 7 atmospheres of oxygen were so rapidly (but approximately equally) inactivated that comparisons were difficult. At 26°, however, there was no appreciable inactivation with time. Enzyme activity was unaffected by 3 to 4 hours exposure to 7 atmospheres of oxygen at this temperature. Lactic dehydrogenase of *Echerichia coli*, on the other hand, was stable at 38° and there was no decrease in activity by exposure for 3 hours to 7 atmospheres of oxygen.

Exposure to 7 atmospheres of oxygen for 4 hours at 38° did not affect the acid phosphatase activity of a rat kidney homogenate.

Phosphorylation of glucose by rabbit kidney extract was unaffected by high pressure of oxygen. The amount of glucose phosphorylated during 1 hour of equilibration was determined. No difference was found between the activity in air and under 7 atmospheres of oxygen.

Table I lists the enzyme systems found to be inactivated by exposure to oxygen at high pressure, the enzyme preparations in which they were studied, and the conditions of the preliminary exposure to oxygen. In each experiment, the activity of the original enzyme preparation was determined. No decrease in activity, or only a small one, was found in the controls. The activities of the preparations exposed to oxygen at high pressure are, therefore, expressed as per cent of the activity of the controls. In all cases the rate of inactivation was slow; several hours exposure to 7 atmospheres of oxygen were necessary to obtain a marked decrease in activity.

Table II gives a list of the enzymes studied in this paper and in the previous papers of this series (11–13). Inactivation by oxygen is indicated in the second column. The rate of inactivation by oxygen has been

**TABLE I**  
*Enzymatic Reactions Inhibited by Exposure to 7 Atmospheres of Oxygen at 38°*

Enzyme	Enzyme preparation (rat tissue)	Exposure period	Activity*	
		<i>hrs.</i>	<i>per cent of control</i>	
Malic dehydrogenase	Liver homogenate	2	97 (a)	
		2	88 "	
		4	49 "	
	Kidney "	2	100 "	
		4	20 "	
	Brain "	2	39 (b)	
Pyruvic oxidase	Liver slices	2	85 (c)	
Choline dehydrogenase		2	57 (b)	
		4	0 (c)	
		4	2 (a)	
		Glutamic acid dehydrogenase	4	52 "
			Kidney "	4
	4	8 "		
$\alpha$ -Ketoglutaric oxidase	" "	2	36 (c)	
		2	51 "	

\* Controls maintained in (a) N<sub>2</sub>, (b) air, (c) 1 atmosphere of O<sub>2</sub>.

**TABLE II**  
*Correlation between Susceptibility to Action of Oxygen and Presence of Essential Sulfhydryl Groups*

Enzymes		Inactivation by oxygen	Essential —SH groups
Oxidative	d-Amino acid oxidase	+	+
	Choline dehydrogenase	+	+
	l(+)-Glutamic dehydrogenase	+	+
	$\alpha$ -Ketoglutaric oxidase	+	+
	Malic dehydrogenase	+	+
	Pyruvic oxidase	+	+
	Succinic dehydrogenase	+	+
	Xanthine oxidase	+	?
	Cytochrome c	—	—
	" oxidase	—	—
Non-oxidative	Lactic dehydrogenase	—	—
	Choline acetylase	+	+
	Carbonic anhydrase	—	—
	Catalase	—	—
	Cholinesterase	—	+
	Pepsin	—	—
	Acid phosphatase	—	—

The evidence for the presence or absence of essential —SH groups is from the papers by Barron and Singer (5, 8, 9), except for choline acetylase which was studied by Nachmansohn and Machado (10). No information on xanthine oxidase is available.

found to vary markedly, depending on the particular enzyme studied and on the type of enzyme preparation used. In Table II, however, all enzymes which were affected by oxygen under the circumstances of our experiments have been classified as inactivated by oxygen.

The presence or absence of essential sulfhydryl groups in the enzymes is also indicated in Table II. The information regarding the presence of essential sulfhydryl groups has been obtained from the literature. In the case of xanthine oxidase, the question of the possible presence of essential sulfhydryl groups has been left open. Experiments by Dixon and Keilin (14), showing that xanthine oxidase of milk is irreversibly inactivated by cyanide, suggest that the activity of this enzyme is independent of sulfhydryl groups. However, we have found no reference in the literature to a study of the effect on xanthine oxidase of agents known to oxidize or combine with sulfhydryl groups.

#### DISCUSSION

The evidence reported in this paper gives considerable support to the view that inactivation by oxygen is a general property of sulfhydryl enzymes. Of the enzymes studied, only one stated to contain essential —SH groups, *viz.* cholinesterase, was resistant to the action of oxygen. Of the enzymes which are not considered sulfhydryl enzymes none was found to be inactivated by oxygen.

The mechanism of oxygen inactivation of sulfhydryl enzymes has been studied in the case of succinic dehydrogenase (3). It was shown that succinic dehydrogenase inactivated by oxygen is reactivated by cysteine or reduced glutathione. Furthermore, malonate and, to a lesser degree, succinate protect the enzyme from the action of oxygen, as they do against the action of oxidized glutathione (15). It was concluded that oxygen, like oxidized glutathione, oxidizes the essential sulfhydryl groups to the inactive form. No such study has been made in the case of other sulfhydryl enzymes, but it seems reasonable to assume that the inactivating effect of oxygen is similar.

In general, the action of oxygen is slow, at least in intact tissue and in the enzymes so far studied. Thus it has not been possible in the study of oxygen poisoning presented in this series of papers to correlate enzyme inhibition with the rapid onset of symptoms exhibited in the intact rat or mouse when exposed to oxygen at high pressure.

The finding that two enzymes, *D*-amino acid oxidase and choline acetylase (12, 13), are rapidly inactivated by oxygen in homogenates or extracts, while they are resistant in intact tissue, is interesting. It illustrates how an enzyme, because of its topical situation in the cell or because of the *milieu interieur*, is protected against deleterious agents. The mechanism

of such protection remains unexplained. However, in one enzyme, succinic dehydrogenase, a protective action of substrate has been demonstrated (3).

It was concluded in a previous paper (16) that the rapid onset of symptoms of oxygen poisoning in the intact animal exposed to 8 atmospheres of oxygen could not be explained by a general inactivation of tissue enzymes resulting in a marked decrease in total oxygen uptake. However, the possibility that enzymatic inhibition plays some part in this acute phase was not completely excluded. It is conceivable that some essential enzyme system unlike most enzymes is particularly susceptible to the action of oxygen. The inactivation of such an enzyme system might explain the symptoms of oxygen poisoning in the intact animal. The experiments reported here on phosphorylation of glucose in kidney extract tend to exclude the possibility that enzymatic phosphorylating mechanisms are peculiarly susceptible to oxygen.

#### SUMMARY

1. In a series of enzymes, those containing essential sulfhydryl groups (with one exception) were found to be inactivated by oxygen; those not containing such groups were not inactivated.

2. This evidence indicates that inactivation by oxygen is a general property of sulfhydryl enzymes.

3. The chemical mechanism of this inactivation is presumed to be an oxidation of the essential sulfhydryl groups to an inactive form.

4. Phosphorylation of glucose in kidney extract was not rapidly affected by oxygen at high pressure, indicating that phosphorylating enzymes are not peculiarly susceptible to oxygen.

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# AN ENZYME IN ANIMAL TISSUES CAPABLE OF HYDROLYZING THE PHOSPHORUS-FLUORINE BOND OF ALKYL FLUOROPHOSPHATES

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A study of the inhibition of plasma, red blood cell, and brain cholinesterase of the rabbit, monkey, and man by diisopropyl fluorophosphate has been reported previously (1). This study related the *in vitro* sensitivities of the various cholinesterases to their inhibition *in vivo*; that is, after inhalation of the vapor or intravenous injection of the compound.

Interest in a possible destruction (detoxification) of diisopropyl fluorophosphate by the animal organism arose because it had been noted that in the presence of serum or plasma and diisopropyl fluorophosphate in a concentration of  $10^{-2}$  mole per liter there occurred a rather large production of  $\text{CO}_2$  from the bicarbonate medium. This did not occur at the same concentration of the fluorophosphate in the absence of serum. Thus, acid was being released by the action of plasma on diisopropyl fluorophosphate. Investigation showed that there was present in the plasma and tissues of the rabbit and of man a substance capable of catalyzing the hydrolysis of diisopropyl fluorophosphate. This substance was shown to be enzymic in nature and at least partially responsible for the *in vivo* destruction of injected diisopropyl fluorophosphate.

## EXPERIMENTAL

*Measurement of Fluorophosphate-Hydrolyzing Activity*—Blood was treated with heparin to prevent clotting. Plasma was used as such or suitably diluted with 0.025 M sodium bicarbonate. Red cells were washed twice with 5 to 10 volumes of saline and diluted four times with 0.025 M bicarbonate. Muscle and brain were carefully removed, freed of any coagulated blood, homogenized in the Waring blender with 9 times their weight of 0.025 M bicarbonate, and filtered through coarse filter paper. Heart, lung, liver, and kidney were perfused with saline in order to reduce the quantity of blood in these tissues, homogenized, and filtered as above. The substrates in these experiments were the various fluorophosphate esters freshly dissolved in 0.025 M bicarbonate. 3 cc. of such a solution containing

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0.035 mm of fluorophosphate were placed in the large well of a Warburg flask. 0.5 cc. of the enzyme (plasma or tissue extract, suitably diluted) in 0.025 M sodium bicarbonate was placed in one side arm. 0.5 cc. of 0.025 M bicarbonate was placed in the other side arm. When the action of inhibitors was studied, the 0.5 cc. of bicarbonate was replaced by an equal volume of solution of the inhibitor dissolved in 0.025 M bicarbonate. A control vessel contained 0.5 cc. of sodium bicarbonate instead of the enzyme solution in order to measure the extent of hydrolysis of the fluorophosphate in water. The solutions were equilibrated at 38° with a mixture of 95 per cent N<sub>2</sub> and 5 per cent CO<sub>2</sub> to give a bicarbonate buffer at pH 7.4. The reaction was started by tipping the contents of the side arms into the main chamber of the vessel. Liberation of CO<sub>2</sub> was taken as evidence of the production of H<sup>+</sup>. Readings were taken at 10 minute intervals and activities calculated from the initial reaction velocity of the straight line portion of the reaction curve.

In some experiments the reaction was followed to completion in order to determine the degree of liberation of hydrogen, phosphate, and fluoride ions. Inorganic phosphate was determined on centrifuged aliquots of the trichloroacetic acid-treated mixtures by the method of Fiske and Subbarow (2). Fluoride was determined by the method of Milton *et al.*<sup>1</sup> For the fluoride determination the reaction mixture was treated with excess silver nitrate in order to precipitate the protein and any inorganic phosphates. After centrifugation, the excess silver in the filtrate was removed by the careful addition of sodium chloride solution, centrifuged, and an aliquot of the filtrate used for the fluoride analysis. The method used was a modification of the thorium nitrate titration with the dye, solochrome blue. Fluoride prevents the formation of the blue color produced by the interaction of thorium nitrate and the dye. Thus, the quantity of thorium nitrate used in the titration is a function of the quantity of fluoride present in the solution. The concentration of fluoride was estimated from a standard curve constructed from data obtained by titration with thorium nitrate in the presence of known quantities of fluoride.

*Evisceration Procedure*—Rabbits were eviscerated under nembutal anesthesia<sup>2</sup> (50 mg. per kilo of nembutal intraperitoneally). The entire gastrointestinal tract and spleen were removed after the portal vein and hepatic artery were securely ligated. The hepatic veins were allowed to remain patent. It has been satisfactorily demonstrated that preparations of this type are similar functionally to hepatectomized animals (3). The abdominal wound was sutured and the animal kept warm by means of the heat from an electric bulb.

<sup>1</sup> Milton, R., Liddell, H. F., and Chivers, J. E.

<sup>2</sup> The author is indebted to Captain J. Tepperman, Medical Corps, for his demonstration of the evisceration technique.

*Measurement of Cholinesterase Activity*—The method used for the estimation of cholinesterase activities in the plasma and various tissues has been described in a previous report (1). Heparin did not affect cholinesterase activities. In the measurement of electric eel<sup>3</sup> cholinesterase activity, it was noted that the high dilutions of the stock solution necessary to measure its activity in the Warburg apparatus caused a rather rapid inactivation of the enzyme; the reaction was not of zero order. It was found that 0.1 per cent gelatin prevented this;  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  were without effect (Fig. 1).

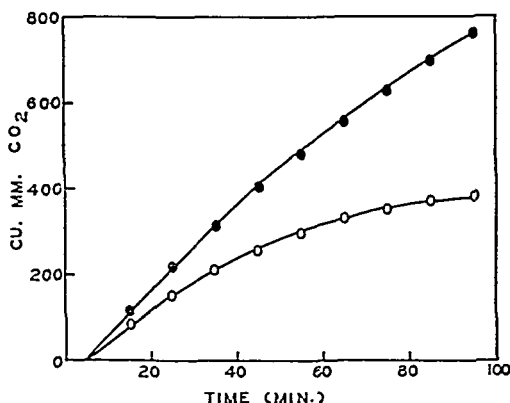


FIG. 1. The effect of gelatin on the spontaneous loss of purified eel cholinesterase activity at high dilutions; O = enzyme activity of eel cholinesterase at a dilution of 1:1600; ● = activity of eel cholinesterase at a dilution of 1:1600, in the presence of 0.1 per cent gelatin.

### Results

*Catalytic Hydrolysis of Diisopropyl Fluorophosphate by Rabbit Plasma and Rabbit and Human Tissue Extracts*—Table I shows the liberation of  $\text{H}^+$  and  $\text{F}^-$  resulting from the action of rabbit plasma on diisopropyl fluorophosphate. No phosphate ion was produced; this indicated that the diisopropyl ester linkages were not split. It may be seen that the production of  $\text{H}^+$  and  $\text{F}^-$  in the presence of plasma was much greater than in the presence of a control bicarbonate solution, pH 7.4 and 38°. The results also show that the liberated  $\text{F}^-$  accounts quantitatively for a reaction in which 1 mole of the fluorophosphate produces 1 mole of  $\text{F}^-$ . The  $\text{H}^+$  production, as measured by  $\text{CO}_2$  liberation, was 1.49 moles per mole of the fluorophosphate. The total acid liberated may be explained by the forma-

<sup>3</sup> The eel cholinesterase was kindly furnished by Dr. D. Nachmansohn, Columbia University, and was prepared from the electric organ.

tion of hydrogen fluoride and the dissociation of the resultant diisopropyl phosphoric acid. A comparison with the theoretical  $H^+$  that could be produced is difficult to make, due to the inherent errors in the method and the presence of plasma protein in relatively high concentration. That there was no cleavage at the isopropyl linkages is indicated by the fact that no inorganic phosphate was produced.

In order to determine whether the factor capable of accelerating the hydrolysis of diisopropyl fluorophosphate was present elsewhere than in

TABLE I

*Effect of Rabbit Plasma on Hydrolysis of Diisopropyl Fluorophosphate*

The control vessels contained 0.035 mm of diisopropyl fluorophosphate in bicarbonate buffer, pH 7.4. Others contained in addition 0.5 cc. of rabbit plasma. The data reported for plasma are not corrected for control hydrolysis in water. The values are expressed in mm produced per mm of diisopropyl fluorophosphate.

Time	CO <sub>2</sub> production in presence of		Fluoride production in presence of		PO <sub>4</sub> <sup>3-</sup> production in presence of	
	Water	Plasma	Water	Plasma	Water	Plasma
<i>min.</i>						
20	0.01	0.34				
30	0.02	0.53	0.03	0.36		
40	0.04	0.70				
60	0.05	1.01	0.08	0.54		
90	0.08	1.31				
120	0.10	1.43	0.07	0.83		
130	0.09	1.44				
140	0.10	1.46	0.07	0.99		
150	0.11	1.48				
160	0.11	1.49	0.08	1.01	0.0	0.001

plasma, extracts of the tissues from rabbits and of human autopsy<sup>1</sup> material were studied. Table II shows the activities of 1:10 extracts of liver, kidney, muscle, lung, heart, and brain made with 0.025 M sodium bicarbonate.

The average activities of six rabbit plasmas and red blood cells and five human plasmas and red blood cells are included for comparison. It may be seen that kidney and liver extracts of both the rabbit and the human are particularly active.

*Nature of Catalysis of Diisopropyl Fluorophosphate Hydrolysis by Plasma and Tissue Extracts*—Table III summarizes the results of experiments devised to answer the question of whether the observed splitting of diisopropyl fluorophosphate by plasma and various tissue extracts is enzymic

<sup>1</sup> The human tissues were specimens from an autopsy and were kindly furnished by Dr. A. Rich, the Johns Hopkins Hospital.

in the usually accepted sense, that is, catalytic and protein or protein-like in behavior, or whether there is present some simple organic or inorganic

TABLE II

*Hydrolysis of Diisopropyl Fluorophosphate by Tissue Extracts*

The velocity of hydrolysis of diisopropyl fluorophosphate was determined as c.mm. of CO<sub>2</sub> liberated in 30 minutes from a bicarbonate buffer, pH 7.4, by 0.5 cc. of a 1:10 tissue extract. The plasma and red blood cell activities were calculated from the activities determined experimentally on more concentrated solutions and also expressed as those of a 1:10 dilution. All data are corrected for the hydrolysis of the diisopropyl fluorophosphate in water.

Tissue	Velocity of hydrolysis by	
	Rabbit	Human
	c.mm. CO <sub>2</sub>	c.mm. CO <sub>2</sub>
Liver	274	457
Kidney .	187	319
Small intestine.	129	
Plasma .	127	19
Lung	55	50
Heart .	30	
Brain .	20	29
Muscle .	12	40
Red cells .	9	13

TABLE III

*Nature of Hydrolytic Action of Rabbit Plasma and Liver Extracts on Diisopropyl Fluorophosphate*

Enzyme source	Treatment	Per cent of original enzyme activity
Liver	Incubation, 38°	100
"	" 50° (5 min.)	71
"	" 60° (5 " )	6
"	" 70° (5 " )	2
Plasma	" 80° (3 " )	0
"	Dialysis, 24 hrs.	109
"	+ NaF (10 <sup>-2</sup> M)	100
"	+ iodoacetate (10 <sup>-2</sup> M)	92
"	+ iodosobenzoate (10 <sup>-3</sup> M)	95
"	+ mercuric ion (10 <sup>-3</sup> M)	8
"	+ " " (10 <sup>-1</sup> M)	29

compound with which diisopropyl fluorophosphate reacts stoichiometrically.

As may be seen, the inactivation of liver extract at moderately high temperatures is typical of an enzyme. Thus, incubation for 5 minutes at 50° resulted in a decrease to 71 per cent of the activity at 38°.

Rabbit plasma was dialyzed against 10 times its volume of saline with ten changes of the outer solution during a period of 24 hours. The type of cellophane membrane used (Visking) was permeable to inorganic ions and organic compounds of low molecular weight. After dialysis, the solution inside the membrane possessed the same activity as before dialysis. This indicated that there were no readily dialyzable components acting as enzymes.

The effect of several typical enzyme inhibitors on the activity of rabbit plasma was studied. Thus fluoride, iodoacetate, and iodosobenzoate did not greatly affect the activity of the factor which is responsible for diisopropyl fluorophosphate hydrolysis. However, mercuric ion, a general enzyme inhibitor, reduced the activity of the diisopropyl fluorophosphate-hydrolyzing enzyme in human plasma.

Since the possibility existed that diisopropyl fluorophosphate reacted non-specifically with proteins, crystalline serum albumin<sup>5</sup> (final concentration 0.63 per cent) was mixed with diisopropyl fluorophosphate in the Warburg vessel. No carbon dioxide was liberated beyond that found in the control. At the end of 1 hour, analysis showed no fluoride ion beyond that present in the control. Similar experiments were carried out with several amino acids; 0.018 mM each of glycine, tyrosine, serine, arginine, glutamic acid, and cysteine was mixed with 0.035 mM of diisopropyl fluorophosphate at pH 7.4. Again no indication of a reaction was evident, as shown by the absence of CO<sub>2</sub> liberation or fluoride ion production. Similar negative results were obtained with several carbohydrates and glycerol.

Further confirmation that the hydrolysis of diisopropyl fluorophosphate was not a non-specific protein effect was the absence of a constant ratio between tissue enzyme activity and protein content. Thus, Table IV shows that the enzyme activity per mg. of protein N of the rabbit kidney extract was 370, that of the liver 294, and that of the muscle 1.

In order to determine whether the phosphorus-containing moiety of diisopropyl fluorophosphate reacts to form a firmly linked compound with the enzyme protein, the following experiment was performed. Serum was allowed to react with diisopropyl fluorophosphate to complete hydrolysis (160 minutes). Trichloroacetic acid was added in the cold to precipitate protein and the filtrate analyzed for organic acid-soluble phosphorus. Within experimental error all of the P due to the diisopropyl fluorophosphate was found to be present in the form of organic acid-soluble phosphorus. Again, no production of inorganic phosphate could be observed.

In order to show a proportional relationship between reaction velocity

<sup>5</sup> The crystalline serum albumin was obtained through the courtesy of Dr. E. J. Cohn, Harvard University.

and enzyme concentration, various dilutions of rabbit plasma were used as a source of the fluorophosphate-hydrolyzing enzyme; the concentration of the fluorophosphate was held constant (0.035 mM per 4.0 cc.). Fig. 2

TABLE IV

*Relationship between Enzyme Activity and Protein Content of Rabbit Tissues*

Protein N is determined as the difference between total N and non-protein N in terms of mg. of N per 0.5 cc. of a 1:10 extract of tissue or dilution of plasma or red cells.

Tissue	Enzyme activity per mg. of protein N
Kidney.....	370
Liver.....	294
Plasma.....	22
Lung.....	16
Heart.....	12
Brain.....	9
Red cells.....	2
Muscle.....	1

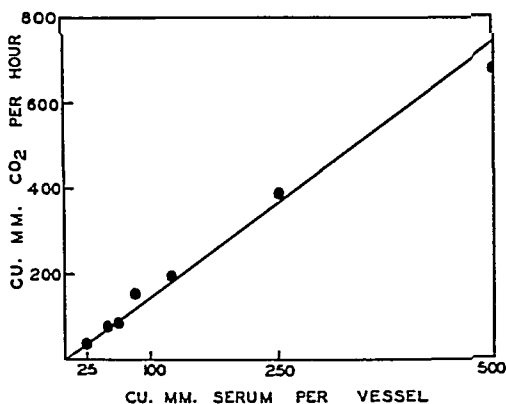


FIG. 2. Relationship between reaction velocity and the concentration of the fluorophosphate-hydrolyzing enzyme in rabbit plasma.

shows that there is a direct proportionality, within experimental error, between reaction velocity and enzyme concentration for almost a 20-fold dilution of the enzyme.

*Hydrolysis of Related Fluorophosphates*—In order to determine whether the *in vitro* mechanism which has been described above holds as well for the related alkyl esters of fluorophosphoric acid, the hydrolyses of the dimethyl, diethyl, and ethylmethyl esters by tissue extracts were studied.

Table V shows the relative enzymic hydrolysis by rabbit plasma and liver extracts of these compounds. In the hydrolyses of these related alkyl esters, fluoride but not phosphate was liberated. This indicated that cleavage occurred at the phosphorus-fluorine bond only.

*Loss of Toxicity of Mixtures of Diisopropyl Fluorophosphate and Plasma*—An aqueous solution of diisopropyl fluorophosphate was incubated with an equal volume of rabbit plasma for 30 minutes at 38° and injected in amounts equivalent to a dose of 1.5 mg. per kilo of diisopropyl fluorophosphate. The injection of this mixture led to no deaths or symptoms of any kind in six out of six rabbits. Injection of diisopropyl fluorophosphate of the same concentration and incubated with phosphate buffer at pH 7.4

TABLE V  
*Hydrolysis of Related Fluorophosphates*

The activities were determined as c.mm. of CO<sub>2</sub> liberated per minute when 0.5 cc. of 1:1 rabbit plasma or 1:10 rabbit liver extract was used in the standard hydrolysis mixture containing 0.035 mM of the dialkyl fluorophosphate in a bicarbonate buffer, pH 7.4. The hydrolysis of the fluorophosphate in water was determined by substituting bicarbonate solution for the enzyme.

Fluorophosphate	Hydrolysis in water	Enzyme activity of	
		Plasma	Liver extract
Dimethyl fluorophosphate . . . . .	10	114	73
Diethyl " . . . . .	2	77	97
Diisopropyl fluorophosphate . . . . .	1	15	22
Ethylnmethyl " . . . . .	2	14	51

for 30 minutes at 38° caused severe symptoms and killed six out of six rabbits within 10 minutes.

*Relationship between Cholinesterase and Diisopropyl Fluorophosphate Hydrolysis*—Cattell reported<sup>6</sup> that a cholinesterase preparation was capable of destroying diisopropyl fluorophosphate. In order to demonstrate that extracts showing cholinesterase activity need not necessarily have the capacity of hydrolyzing diisopropyl fluorophosphate, the action of a purified electric eel cholinesterase preparation on the fluorophosphate was studied (Table VI).

Purified cholinesterase, in a dilution of 1:400 in the final hydrolysis mixture, liberated 2580 c.mm. of CO<sub>2</sub> in 30 minutes from 0.015 M acetylcholine but showed no ability to hydrolyze diisopropyl fluorophosphate. It may also be seen that inactivation of rabbit plasma cholinesterase by pre-treatment with a very low concentration of diisopropyl fluorophosphate,

<sup>6</sup> Cattell, McK.



although it inhibited all of the cholinesterase activity, did not affect the ability of the plasma to hydrolyze added diisopropyl fluorophosphate.

*In Vivo Demonstration of Rôle of Liver in Detoxification of Diisopropyl Fluorophosphate*—Table II shows that on a unit weight basis the liver has a greater enzyme capacity for hydrolyzing diisopropyl fluorophosphate than any other tissue tested. In addition, the weight of the liver in the rabbit is approximately 45 gm. per kilo of body weight as compared with the kidneys, 8 gm. per kilo. This indicates that the liver as an organ may play 5 times as great a rôle in the destruction of the fluorophosphate. Rabbits were eviscerated and control blood samples withdrawn from the femoral artery or by cardiac puncture. The animal was injected intravenously (ear vein) with a freshly prepared solution of diisopropyl

TABLE VI

*Relationship between Cholinesterase and Diisopropyl Fluorophosphate Hydrolysis*

The cholinesterase activity was determined as c.mm. of CO<sub>2</sub> produced in 30 minutes from a solution of acetyl holine (see (1)). The activity of the diisopropyl fluorophosphate-splitting enzyme was determined as c.mm. of CO<sub>2</sub> produced in 30 minutes in the standard hydrolysis solution previously described.

Source of cholinesterase	Cholinesterase activity	Diisopropyl fluorophosphate-hydrolyzing activity
Electric eel, 0.01 cc.	2580	0
Plasma, rabbit, 0.5 cc. . . .	92	521
“ “ 0.5 “ pretreated with 10 <sup>-3</sup> M diisopropyl fluorophosphate	0	520

fluorophosphate, and after a suitable period of time the animal was sacrificed by the intravenous injection of air and the brain removed. Control rabbits were eviscerated and their brains removed without the injection of the fluorophosphate in order to determine whether operation and the anesthesia affected the brain cholinesterase activity. A dose of 0.1 mg. per kilo of diisopropyl fluorophosphate, equivalent to a dose of 0.13 mg. per kilo on the basis of the weight of the eviscerated animal, was then injected intravenously. Non-eviscerated animals were used as controls and injected with a dose of 0.13 mg. per kilo of diisopropyl fluorophosphate under the same conditions of anesthesia.

The results are shown in Table VII. Non-eviscerated rabbits, injected with 0.13 mg. per kilo of diisopropyl fluorophosphate, showed decreases in plasma cholinesterase averaging 30 per cent of the preinjection value. In the eviscerated animals the plasma cholinesterase activities averaged 27 per cent of normal. Thus, exclusion of the liver from the circulation

did not produce any significant effect on the lowering of the plasma cholinesterase activity after intravenous diisopropyl fluorophosphate.

The activities of the red blood cell cholinesterase in the non-eviscerated animals injected with diisopropyl fluorophosphate averaged 61 per cent of normal, whereas the red cell cholinesterase activities of the eviscerated animals averaged 43 per cent of normal. There appeared to be a significantly greater decrease of red cell cholinesterase activity as the result of the injection of diisopropyl fluorophosphate in the eviscerated animals.

TABLE VII

*In Vivo Effect of Diisopropyl Fluorophosphate on Cholinesterase Activity of Plasma, Red Blood Cells, and Brain of Eviscerated Rabbits*

The rabbits were injected with 0.13 mg. per kilo of the fluorophosphate on the basis of the weight of the eviscerated animal. The non-eviscerated animals were injected with 0.13 mg. per kilo of the fluorophosphate. The brain cholinesterase activities of rabbits that were eviscerated but not injected yielded values similar to those obtained from normal animals.

Time after injection	Non-eviscerated			Time after injection	Eviscerated		
	Per cent of normal cholinesterase activity in				Per cent of normal cholinesterase activity in		
	Plasma	Red cells	Brain		Plasma	Red cells	Brain
<i>min.</i>				<i>min.</i>			
31	42	74	54	30	19	30	14
30	30	54	42	24	32	48	22
30	26		25	22	27		7
30	32	47	32	22	28	26	3
21	25	69	36	17	33	53	6
17	31	62	42	14	25	38	0
17	23	61	21	8	26	30	1
17			32				
Aver- age.....	30	61	36		27	43	8

The activities of the brain cholinesterase in non-eviscerated animals injected with diisopropyl fluorophosphate averaged 36 per cent of normal. In the eviscerated animals injected with an equivalent dose of diisopropyl fluorophosphate, the brain cholinesterase activities averaged 8 per cent of normal. Hence the removal of the liver from the circulation caused a marked *in vivo* reduction of brain cholinesterase activity. It may be noted that the decrease in the brain cholinesterase activity appeared to be independent of the duration of the interval between injection of diisopropyl fluorophosphate and removal of the brain.

*Purification and Properties of Enzyme Responsible for Hydrolysis of Diisopropyl Fluorophosphate*—Since it would be of general interest to know the normal substrate in the body which is acted upon by the enzyme described above, a preliminary attempt was made to purify this enzyme from rabbit kidneys which contain the highest concentration of activity per mg. of protein N (see Table IV) of all the tissues investigated. Table VIII describes the various procedures used in the purification of the enzyme.

TABLE VIII

*Purification of Fluorophosphate-Hydrolyzing Enzyme from Rabbit Kidneys*

The activities were measured as c.mm. of CO<sub>2</sub> produced in 30 minutes by 1 cc. of enzyme solution from the standard hydrolysis mixture.

Fraction	Treatment	Activity per cc	Total N per cc.	Activity per mg total N
		<i>c.mm</i>	<i>mg</i>	<i>c.mm</i>
A	Crude extract with 0.025 M NaHCO <sub>3</sub>	748	2.604	287
B	Adjust A to pH 5.0, discard ppt.	866	1.869	464
C	Filtrate B, pH 5.0, alcohol to 52%; discard filtrate; extract with 0.025 M NaHCO <sub>3</sub>	255	0.196	1300
D	Same as C, but pH 5.4	481	0.174	2760
E	" " " " " 5.9	385	0.196	1965
F	" " " " " 6.3	146	0.179	815
G	Filtrate B, pH 5.0, alcohol to 29%; discard ppt., add alcohol to 52%; discard filtrate; extract ppt. with 0.025 M NaHCO <sub>3</sub>	384	0.216	1780
H	Same as G, but pH 5.4	494	0.134	3690
I	" " " " " 5.9	666	0.185	3600
J	" " " " " 6.3	308	0.174	1770

The highest degree of purification was attained by the following procedure: The kidneys were homogenized in the Waring blender with 5 times their weight of 0.025 M sodium bicarbonate for 1 minute. The pH of the suspension was adjusted to 5.0 and the mixture allowed to stand in the cold for 1 hour. The temperature during all manipulations was kept as close to 5° as was feasible. The precipitate was centrifuged and discarded. The filtrate was rapidly adjusted with 5 per cent sodium carbonate to pH 5.4 and alcohol added in the cold to about 29 per cent. After standing in the cold for a half-hour, the precipitate was removed by centrifugation and alcohol was added to the filtrate to about 55 per cent. The precipitate, which was active, was centrifuged and the alcoholic filtrate discarded. The precipitate was extracted with 0.025 M sodium bicarbonate (one-half

the volume of the original bicarbonate used for the kidneys) and the suspension centrifuged. The clear, red-colored filtrate was preserved with toluene and stored in the ice box.

The original filtered bicarbonate kidney extract had an activity of 287 (c.mm. of  $\text{CO}_2$  liberated in 30 minutes by 1.0 cc. of the enzyme solution) per mg. of total N; the final extract had an activity of 3690 and was therefore 13 times as pure as the original extract on the basis of N content.

Table IX shows the relative sensitivity to pH of the enzyme solution purified in the above manner. It may be seen that below a pH of 7.0 the

TABLE IX

*Effect of pH on Stability of Purified Fluorophosphate-Hydrolyzing Enzyme*

The purified enzyme solution (Fraction H, Table VIII) was adjusted to the proper pH with dilute acetic acid or sodium carbonate and allowed to stand for 18 hours at ice box temperature. The activities were determined on 0.5 cc. of a 1:5 dilution of the enzyme, brought to pH 7.4, and expressed as c.mm. of  $\text{CO}_2$  liberated in 30 minutes.

pH	Activity per mg. total N
	c.mm.
4.1	0
4.6	41
5.0	320
5.2	1025
5.9	1890
6.9	2420
7.4	2080
8.2	2060
9.4	1980
9.8	2090

activity is destroyed to a great extent on standing. At the more alkaline pH's the activity did not decrease to any great extent.

Table X shows that dialysis of the purified enzyme solution against 0.025 M sodium bicarbonate for 7 hours resulted in the loss of 12 per cent of its original activity. This loss of activity could be partially restored by the addition of some of the dialysate to the dialyzed solution. It may also be seen that prolonged dialysis (2 days) of the purified enzyme against many changes of 0.01 M phosphate buffer, pH 7.4, containing 0.9 per cent saline resulted in a 28 per cent loss of activity. This loss of activity could be replaced almost completely by 0.01 M  $\text{Ca}^{++}$  or 0.01 M  $\text{Mg}^{++}$  (as chlorides). These two ions may be considered as activators of the enzyme. These results may be compared with those reported in a previous section on the dialysis of rabbit plasma. It is not surprising that purification of the en-

zyme resulted in a greater sensitivity to dialysis or, as will be shown subsequently, to the action of inhibitors.

Since it is possible that the kidney enzyme preparations also possessed phosphatase activity, the action of the crude (Fraction A, Table VIII) as well as the purified (Fraction H, Table VIII) enzymes was studied at optimum conditions of pH (8.7) and substrate, magnesium, and glycine concentrations. Inorganic phosphate was determined by the method of Fiske and Subbarow (2). Phosphatase activities were compared with the activities of the same preparations when diisopropyl fluorophosphate was used as a substrate and  $\text{CO}_2$  production used as an index of enzyme activity.

TABLE X

*Effect of Dialysis on Purified Fluorophosphate-Hydrolyzing Enzyme*

Treatment	Per cent of original enzyme activity
Dialyzed Enzyme I*	88
“ “ “ + dialysate†	94
“ “ II‡	72
Enzyme II + calcium (0.01 M)	96
Dialyzed Enzyme II + calcium (0.01 M)	94
Enzyme II + magnesium (0.01 M)	107
Dialyzed Enzyme II + magnesium (0.01 M)	87

\* Dialyzed 7 hours; 10 cc. of the enzyme solution against 50 cc. of 0.025 M  $\text{NaHCO}_3$ .

† 0.5 cc. of the dialysate added to 0.5 cc. of the dialyzed enzyme.

‡ Dialyzed 48 hours against a total of 2 liters of 0.01 M phosphate buffer, pH 7.4, containing 0.9 per cent saline with twenty changes of the solution.

Table XI shows that there is little phosphatase activity in either the crude or purified preparations. It also indicates that the purification procedure resulted in a loss of phosphatase activity, although the diisopropyl fluorophosphate-hydrolyzing activity was increased considerably. These findings, together with the facts previously reported that the action of the enzyme which splits diisopropyl fluorophosphate does not produce inorganic phosphate, indicate that phosphatase activity is not related to the enzyme activity responsible for the hydrolysis of diisopropyl fluorophosphate.

Table XI also presents the results of the determination of cholinesterase activities with acetylcholine as a substrate, and esterase activities with triacetin, tripropionin, and methyl butyrate as substrates, on both the crude and purified extracts. It may be seen that neither fraction contained any cholinesterase activity. The crude fraction contained esterase activity, which was appreciably reduced in the purified fraction.

TABLE XI

*Relationship between Fluorophosphate-Hydrolyzing Enzyme and Phosphatase, Cholinesterase, and Esterase Activities*

The phosphatase activities are measured by the production of inorganic phosphate from  $\beta$ -glycerophosphate in 1 hour. The activities of esterase and cholinesterase are measured by the production of  $\text{CO}_2$  by 0.5 cc. of the enzyme solution from a bicarbonate buffer, pH 7.4, containing 0.015 mole per liter of acetylcholine, triacetin, tripropionin, or methyl butyrate.

Substrate	Enzyme activity in	
	Fraction A, Table VIII	Fraction H, Table VIII
Diisopropyl fluorophosphate, c.mm. $\text{CO}_2$ .....	388	3610
Acetylcholine, c.mm. $\text{CO}_2$ .....	0	0
Glycerophosphate, mg. $\text{PO}_4$ .....	0.144*	0.080
Triacetin, c.mm. $\text{CO}_2$ .....	288	14
Tripropionin, c.mm. $\text{CO}_2$ .....	510	49
Methyl butyrate, c.mm. $\text{CO}_2$ .....	227	7

\* This quantity of inorganic phosphate accounts for but 4.6 per cent of the available phosphate from glycerophosphate. In a similar experiment with rat intestinal phosphatase, 61 per cent of the available phosphate from glycerophosphate was hydrolyzed in 1 hour.

TABLE XII

*Effect of Inhibitors on Purified Fluorophosphate-Hydrolyzing Enzyme*

The activities were measured as c.mm. of  $\text{CO}_2$  produced in 30 minutes by 0.5 cc. of the diluted enzyme, Fraction H, Table VIII. The inhibitor and enzyme were added simultaneously to the substrate (diisopropyl fluorophosphate) at the start of the experiment.

Inhibitor	Concentration of inhibitor	Per cent of original enzyme activity
	<i>mole per l.</i>	
$\text{Hg}^{++}$ .....	$2 \times 10^{-5}$	0
$\text{Cu}^{++}$ .....	$1 \times 10^{-5}$	53
$\text{Fe}^{+++}$ .....	$1 \times 10^{-3}$	94
Arsenite.....	$1 \times 10^{-3}$	93
Iodoacetate.....	$1 \times 10^{-3}$	89
Iodosobenzoate.....	$1 \times 10^{-3}$	100
Iodine.....	$1 \times 10^{-4}$	57
Fluoride.....	$1 \times 10^{-2}$	106
Cysteine.....	$1 \times 10^{-3}$	93

The effect of inhibitors on the purified enzyme was studied. The results are presented in Table XII. Mercuric and cupric ions inhibited the enzyme activity at low concentrations. Iodoacetate (recrystallized to remove

traces of iodine) caused some inhibition. Iodine was effective as an inhibitor. Arsenite, ferric ion, iodosobenzoate, and cysteine did not affect the enzyme activity in the concentrations employed.

Table XIII shows the extent of inhibition at various concentrations of  $\text{Hg}^{++}$ . It may be seen that a curious phenomenon of acceleration of enzyme activity exists at low concentrations of  $\text{Hg}^{++}$ . Thus at  $2 \times 10^{-5} \text{ M Hg}^{++}$  the enzyme activity was zero, at  $6 \times 10^{-6} \text{ M Hg}^{++}$  it was 240 per cent of the

TABLE XIII  
*Effect of Varying Concentrations of  $\text{Hg}^{++}$  and  $\text{Cu}^{++}$  on Fluorophosphate-Hydrolyzing Enzyme*

Inhibitor	Concentration of inhibitor <i>mole per l</i>	Per cent of original enzyme activity
$\text{Hg}^{++}$	$1 \times 10^{-4}$	0
	$6 \times 10^{-5}$	0
	$2 \times 10^{-5}$	0
	$1 \times 10^{-5}$	63
	$8 \times 10^{-6}$	181
	$6 \times 10^{-6}$	240
	$4 \times 10^{-6}$	136
	$2 \times 10^{-6}$	112
	$1 \times 10^{-6}$	113
$\text{Cu}^{++}$	$1 \times 10^{-4}$	4
	$1 \times 10^{-5}$	53
	$5 \times 10^{-6}$	75
	$1 \times 10^{-6}$	105
	$1 \times 10^{-7}$	110

untreated enzyme, and at  $1 \times 10^{-6} \text{ M Hg}^{++}$  the enzyme activity was essentially the same as that of the untreated enzyme. Cupric ion, although an inhibitor, did not show this accelerating effect.

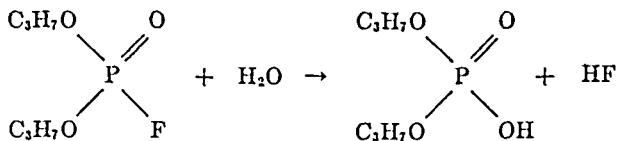
#### DISCUSSION

An examination of the chemical structure of diisopropyl fluorophosphate indicates that there are two possible centers of chemical reactivity. The first is the alkyl ester linkage and the second is the phosphorus-fluorine linkage present in all of the physiologically active members of the alkyl fluorophosphates. The stability of the alkyl ester linkage, such as exists in diisopropyl fluorophosphate, has been demonstrated by the finding<sup>7</sup> that after refluxing 1 equivalent of the fluorophosphate with 4 equivalents of 5 per cent NaOH for 24 hours the distillate from such a mixture failed to

<sup>7</sup> Price, C. C.

give a positive iodoform reaction, which would have been indicative of free isopropyl alcohol. Similar results were reported with 10 per cent alkali for 72 hours. *In vitro* experiments with various esters of phosphoric acid as substrates for phosphatase action (4) indicated that even at optimum pH (8.8) the rate of splitting of such esters as monopropyl phosphoric acid is extremely low. The second linkage which might be involved is the phosphorus-fluorine bond. This is known to be hydrolyzed by water in the various fluorophosphate derivatives. The extent of hydrolysis varies. Thus, the diethyl ester is hydrolyzed to the extent of 24 per cent in 1 hour at 25°, whereas diisopropyl fluorophosphate is hydrolyzed 1 per cent in the same time at the same temperature.<sup>8</sup> It was previously observed that an aqueous solution of diisopropyl fluorophosphate on standing lost some of its capacity to inhibit cholinesterase activity *in vitro* (1).

The data presented show that diisopropyl fluorophosphate is hydrolyzed by a heat-labile, non-dialyzable substance, present in plasma and various tissues of the rabbit and man, to form inorganic fluoride and probably diisopropyl phosphoric acid. The fluoride produced accounted quantitatively for all of the diisopropyl fluorophosphate, whereas no inorganic phosphate was formed. This same reaction takes place, though much more slowly, in aqueous solution at pH 7.4.



The substance responsible for the accelerated hydrolysis of diisopropyl fluorophosphate also splits other dialkyl fluorophosphates at the same linkage at varying rates, thus indicating that it is, so far as this group of compounds is concerned, specific only for the phosphorus-fluorine linkage.

The application of various criteria for enzyme activity to both crude tissue extracts as well as to partially purified preparations from the rabbit kidney has shown that the factor capable of catalyzing the hydrolysis of diisopropyl fluorophosphate is enzymic in nature. Thus, it is heat-labile, difficult to remove by dialysis, sensitive to low pH, and shows the typical behavior of enzymes in the presence of inhibitors. It is not a non-specific protein, as was evidenced by a lack of constancy of the ratio of activity to protein content of the various tissues. The activity, as present in rabbit plasma, is proportional to the enzyme concentration over a wide range of dilutions.

Since it is obvious that this enzyme, normally present in animal tissues,

<sup>8</sup> National Defense Research Council Publication.



has some other substrate, preliminary attempts at purification were made to define if possible its relationship to some known enzyme already described. It was demonstrated that preparations of this enzyme could be concentrated about thirteen times with respect to its ability to hydrolyze diisopropyl fluorophosphate but that such purification resulted in the loss of the phosphatase, esterase, and cholinesterase activities.<sup>2</sup>

The behavior of the enzyme in the presence of various inhibitors is of interest. Thus, in the crude state (plasma and liver extracts) there was no evidence of inhibition of activity by  $10^{-2}$  M iodoacetate, whereas in the partially purified form from rabbit kidneys the enzyme showed some reduction in activity due to  $10^{-2}$  M iodoacetate. Inhibition by iodoacetate and the inhibition of the enzyme by low concentrations of iodine indicate that the sulfhydryl grouping may be associated with the enzyme activity. The enzyme is also inhibited by low concentrations of  $\text{Hg}^{++}$  and  $\text{Cu}^{++}$ .

The acceleration of enzyme activity by certain concentrations of  $\text{Hg}^{++}$  presents an interesting finding similar in nature to that previously reported by Lipmann (5) for the inhibition of glycolysis of crude muscle extracts by  $\text{Cu}^{++}$ . At certain concentrations of metallic ion the enzyme activity is markedly increased over that of the untreated enzyme preparation. This effect may be explained by assuming the presence of some substance in the impure enzyme preparation which acts as an inhibitor of the enzyme activity. Thus, at certain concentrations of  $\text{Hg}^{++}$  this naturally occurring inhibitor is inactivated by the  $\text{Hg}^{++}$ , which, however, is not present in high enough concentrations to inhibit the enzyme, thus leading to an over-all activation of the enzyme.

The enzyme present in plasma is capable of destroying the toxic properties of diisopropyl fluorophosphate *in vitro*. It has also been shown that the enzyme, present in high concentration in the liver, plays a rôle in the detoxification of diisopropyl fluorophosphate; that is, in reducing its effectiveness as an *in vivo* inhibitor of red blood cell and brain cholinesterases. Thus, the inhibitory effect of a given injected dose of the fluorophosphate was more marked on the red cell and brain cholinesterases of the eviscerated rabbit than that obtained with an equivalent dose injected into a non-eviscerated animal. The inhibition of plasma cholinesterase of the eviscerated animal was not different than that obtained in the intact

<sup>2</sup> The question arises whether to assign a name to this enzyme on the basis of its known activity; that is, its ability to catalyze the hydrolysis of the fluorophosphates at the phosphorus-fluorine linkage. Such a name might be "phosphofluorase." The author intentionally has refrained from using any name as he feels that its introduction into the literature would confuse rather than clarify and should await its identification with an enzyme known to act on a substrate normally present in animal tissues.

animal. Certain factors may be concerned in this latter phenomenon: the mode of administration of the fluorophosphate so as to bring it into immediate contact with the plasma cholinesterase; the differing sensitivities of the various cholinesterases to the fluorophosphate; the localization of the fluorophosphate in the brain, due to its high lipid solubility. In view of the fact that complete data are not available to evaluate these factors, no attempt at a rigid explanation can be made.

The finding that uncombined diisopropyl fluorophosphate is destroyed by various tissue extracts emphasizes the conclusions previously reported (1) that the effect of diisopropyl fluorophosphate on cholinesterase is irreversible. If the fluorophosphate and cholinesterase were combined to form a dissociable or easily dissociable complex, then the hydrolysis of any free fluorophosphate due to dissociation would shift the equilibrium so as to liberate completely the active cholinesterase. However, it was shown that rabbits recovering from a sublethal single injection of diisopropyl fluorophosphate showed a regeneration of brain cholinesterase far too slow to be accounted for by the liberation of cholinesterase from a dissociable cholinesterase-fluorophosphate complex.

The present results together with those previously reported (1) yield the following conception of the fate of diisopropyl fluorophosphate in the animal organism: On the one hand, diisopropyl fluorophosphate reacts with cholinesterase in the plasma, red blood cells, and various tissues to produce an irreversible inactivation of these cholinesterases. Concurrently and in competition with this process, the fluorophosphate is destroyed by an enzyme present in various tissues, especially the liver and kidney. It may be seen that the degree of physiological symptoms or the death of the animal is a resultant of the two processes. The present data do not permit a quantitative evaluation of the rôle of each of these processes.

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#### SUMMARY

1. Rabbit and human plasma, red blood cells, and tissues contain an enzyme which accelerates greatly the hydrolysis of the dialkyl fluorophosphates to the dialkylphosphoric acid, hydrogen, and fluoride ions.

2. The enzyme from rabbit kidneys has been concentrated thirteen times, on the basis of total nitrogen content. It is sensitive to heat, acid pH, and low concentrations of iodine,  $\text{Hg}^{++}$ , and  $\text{Cu}^{++}$ ; it shows some inhibition by iodoacetate and is insensitive to fluoride, iodosobenzoate, arsenite, and  $\text{Fe}^{+++}$ . Some loss of activity occurs after dialysis but may

be restored by the addition of the dialysate,  $\text{Ca}^{++}$ , or  $\text{Mg}^{++}$  to the dialyzed solution. The enzyme is not related to phosphatase, cholinesterase, or esterase.

3. The enzyme mechanism is important in the detoxification of diisopropyl fluorophosphate. Liver, which contains relatively high concentrations of the enzyme, plays a significant rôle in the destruction of diisopropyl fluorophosphate in the intact animal.

4. The fate of the dialkyl fluorophosphates in the animal organism is a resultant of the irreversible inactivation of plasma, red cell, and tissue cholinesterases and the detoxification of the fluorophosphate, especially in the liver.

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# A QUANTITATIVE STUDY OF THE PRODUCTS OF FATTY ACID OXIDATION IN LIVER SUSPENSIONS\*

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It is now generally recognized that the complete oxidation of the bulk of the fatty acids in the mammalian organism takes place in what may be regarded as two phases. In the *primary* phase the fatty acids are oxidized in the liver to the ketone bodies. No other intermediates of fatty acid oxidation in the liver have been identified. The liver, however, has only a limited ability to oxidize the ketone bodies further and these substances therefore diffuse into the blood stream. The extrahepatic tissues, especially the kidney and muscles, are capable of completing the oxidation of the ketone bodies to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  and therefore are the site of the *secondary* phase of fatty acid oxidation. The possibility that the extrahepatic tissues are also capable of the "direct" oxidation of fatty acids must of course be left open. Apart from over-all respiratory quotient data, there have been no strikingly decisive experiments reported which prove or disprove "direct" oxidation of higher fatty acids in the extrahepatic tissues. At any rate it is probably accurate to presume that the "diphasic" oxidation of fatty acids outlined above is the mechanism of oxidation of a major share of the fatty acids undergoing oxidative degradation in the organism.

Recently the *secondary* phase of this diphasic process, the oxidation of acetoacetate in the extrahepatic tissues, has been investigated with considerable success by Breusch (2), Wieland and Rosenthal (3), Martius (4), Hunter and Leloir (5), and Buchanan *et al.* (6), who have shown that acetoacetate (possibly after cleavage into 2-carbon fragments) is oxidized through the events of the Krebs tricarboxylic acid cycle.

The *primary* phase of the oxidation occurring in the liver has, however, not been quite as amenable to experimentation, and the mechanism is known only in the grossest of terms. A single fatty acid molecule on oxidation may give rise to more than 1 molecule of ketones (7), and, under

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conditions of fasting or diabetes mellitus, the yield may approach a quantitative conversion of the carbon of the fatty acids to ketones (8, 9). It is certain, however, that this conversion to ketones is not quantitative under all conditions. The possibility that fatty acids may undergo oxidation in the liver by a second pathway not involving the ketone bodies as obligatory intermediates has often been considered (10). The mechanism of such a pathway and its relationship to ketone body formation are obscure.

Recently the author described the preparation of a washed rat liver suspension which is capable of oxidizing the higher fatty acids in the presence of adenosine triphosphate (ATP) and magnesium ions, with the production of acetoacetate (11). This oxidation is uncomplicated by other metabolic activities, since the enzyme suspension has negligible oxygen uptake in the absence of substrate. A quantitative study of the products of enzymatic fatty acid oxidation in these rat liver suspensions shows that all of the carbon of the oxidized fatty acid is recovered as acetoacetate in the *absence* of oxalacetate or oxalacetate precursors. In the *presence* of the latter, part of the carbon is recovered as citric,  $\alpha$ -ketoglutaric, and succinic acids, and in such amounts that it may be concluded that the Krebs tricarboxylic acid cycle accounts for that portion of the oxidative degradation of fatty acids which does not proceed through the formation of acetoacetate.

#### EXPERIMENTAL

*Preparative*—Adenosine triphosphate (ATP) was prepared as previously mentioned (12). Only material approaching analytical purity was used, since impure preparations often contained substances which completely inhibited fatty acid oxidation. Sodium pyruvate was prepared as described by Robertson (13), sodium acetoacetate according to Ljunggren (14). The enzyme suspensions were prepared exactly as described in a previous paper (11) and were used immediately.

*Analytical*—Oxygen uptake was measured manometrically in standard Warburg flasks with alkali and filter paper in the center well. The contents of the side arm were tipped into the main compartment (at zero time), after a 3 minute equilibration period. In some experiments all components were placed in the main compartment and manometric data were extrapolated back to the time of addition of the enzyme. The respiratory quotient was determined by Warburg's direct method (15). Pyruvate was determined according to Friedemann and Haugen (16), after decarboxylation of acetoacetate by aniline (to minimize interference by acetoacetate). Acetoacetate was determined manometrically with aniline citrate, or colorimetrically as acetone by a modification of the method of Greenberg and Lester (17). In media containing oxalacetate this substance was first decomposed into pyruvate and  $\text{CO}_2$  by incubation with 5

per cent  $\text{CuSO}_4$  (18) to eliminate interference in the manometric determination of acetoacetate. Several modifications of the colorimetric pentabrom-acetone method for citric acid were used; the most useful and reliable was found to be that of Speck, Moulder, and Evans (19). The sum of  $\alpha$ -ketoglutarate plus succinate in the presence of malonate was determined enzymatically, according to the procedures of Krebs and Eggleston (20).

*Formation of Acetoacetate during Octanoate Oxidation*—In experiments designed to determine in a quantitative manner the yield of acetoacetate

TABLE I

*Respiratory Quotient and Acetoacetate Formation during Octanoate Oxidation*

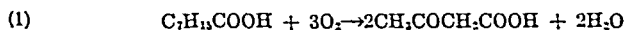
The main compartment of the Warburg vessel contained 0.60 ml. of enzyme, 0.20 ml. of phosphate buffer, pH 7.5 (0.008 M),\* 0.20 ml. of  $\text{MgSO}_4$  (0.005 M), 0.20 ml. of sodium malonate† (0.01 M), 0.40 ml. of Na adenosine triphosphate (0.0013 M). The side arm contained 0.40 ml. of sodium octanoate (0.001 M) or  $\text{H}_2\text{O}$ . Time, 27 minutes, temperature  $31^\circ$

Octanoate	O <sub>2</sub> uptake	Acetoacetate formation	$\frac{\text{Acetoacetate}}{\text{O}_2}$	R.Q.
	c. mm.	c. mm.		
—	1	3		
+	101	64.6	0.62	0.12
+	103	69.7	0.65	0.08
+	105	69.1	0.64	0.03
Calculated, equation (1)			0.67	0.00

\* The figures in parentheses indicate the concentration of the component in the complete reaction medium. This notation is used in Tables I to VII and in Fig. 1.

† Malonate (0.01 M) was present in the enzyme media in all experiments reported in this paper. It served the double purpose of inhibiting the slight residual respiration of the liver suspensions and of causing the accumulation of certain intermediates of the Krebs cycle in those experiments in which this end was desired.

during the oxidation of octanoate by the washed rat liver suspension described in the preceding paper (11), the oxygen uptake data and the yields of acetoacetate suggested that the oxidation was proceeding in such a way that it could be expressed by the equation



It can be seen by inspection of the equation that the R.Q. of this reaction is zero, and the ratio of acetoacetate formed to oxygen taken up (in terms of c. mm. of  $\text{O}_2$  and acetoacetate) is 0.67. In Table I are shown data collected on typical experiments; the R.Q., measured by Warburg's direct method, approaches the theoretical zero, and the ratio of acetoacetate formed to oxygen used approximates 0.67. The formation of 2 molecules

of acetoacetate from 1 of octanoate in these washed preparations, which are devoid of other metabolic events, represents a quantitative conversion of the carbon of the fatty acid into acetoacetate.

The presence or absence of alkali in the center well had no effect on the acetoacetate formation, showing that  $\text{CO}_2$  probably had no influence on the course of the oxidation and that the use of Warburg's direct method was therefore valid.

The calculated oxygen uptake for the complete conversion of the octanoate present to acetoacetate, according to equation (1) in the experiments in Table I, is 134.4 c.mm. Values of 127, 128, and 137 were obtained in parallel experiments when the oxidation was allowed to go to completion. Octanoate determinations were not made routinely, but in a similar, large scale experiment the total volatile fatty acids remaining after complete oxidation were determined by distillation and titration; the distillate contained but 3 per cent of the amount present before oxidation.

Acetoacetate was the sole keto acid accumulating during octanoate oxidation. This was indicated not only by the stoichiometric relationships but also by the fact that identical analytical values for acetoacetate were obtained (a) by the manometric aniline citrate method (specific for  $\beta$ -keto acids) and (b) by the colorimetric determination of acetone formed in the catalytic decarboxylation of acetoacetate by aniline by a method (17) found by the author to give color only with *neutral* carbonyl compounds under the conditions of these experiments. Only traces of color were given in the Friedemann and Haugen extraction method (16) for keto acids when applied to the media following treatment with aniline acetate. Therefore no  $\alpha$ -keto acids accumulated during the course of octanoate oxidation.

The ratio of acetoacetate formation to oxygen uptake was essentially constant throughout the oxidation (Fig. 1).

Acetoacetate was not significantly attacked by the enzyme (see Table II), either in the presence or absence of fumarate or oxalacetate and ATP.

There was no evidence that  $\beta$ -hydroxybutyrate was formed during the oxidation; the compound was readily oxidized by the enzyme preparation, but not at a rate consistent with the view that it was an intermediate in acetoacetate formation in these experiments.

The essentially quantitative agreement between  $\text{O}_2$  uptake and ketone formation in these preparations is in contrast to the data of Leloir and Muñoz (21), who found an unaccountably large uptake of  $\text{O}_2$  and formation of  $\text{CO}_2$  in their preparations during butyrate oxidation.

*Formation of Acetoacetate during Pyruvate Oxidation*—One of the metabolic reactions of pyruvic acid is the formation of acetoacetate. This reaction is demonstrated most readily in the liver, in which the formation



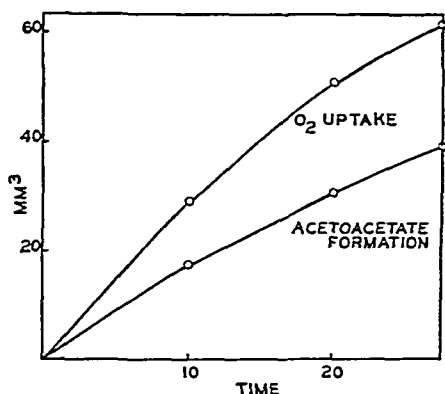


FIG. 1. The rate of acetoacetate formation and oxygen uptake during octanoate oxidation. Main compartment, 0.30 ml. of enzyme, 0.10 ml. of  $\text{MgSO}_4$  (0.005 M), 0.10 ml. of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.10 ml. of phosphate buffer (0.008 M), 0.10 ml. of malonate (0.01 M), 0.10 ml. of adenosine triphosphate (0.0015 M). Side arm, 0.20 ml. of octanoate (0.001 M) or  $\text{H}_2\text{O}$ . Blank values (no substrate) subtracted in above curves. Reaction stopped for acetoacetate analyses at times designated by tipping 0.20 ml. of 20 per cent trichloroacetic acid into the medium from the second side arm. Temperature  $31^\circ$ .

TABLE II  
*Non-Oxidation of Acetoacetate*

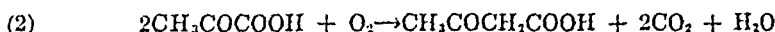
The main compartment contained 0.60 ml. of enzyme, 0.20 ml. of phosphate buffer, pH 7.7 (0.008 M), 0.20 ml. of sodium malonate (0.01 M), 0.20 ml. of  $\text{MgSO}_4$  (0.005 M), 0.20 ml. of adenosine triphosphate (0.0013 M), 0.20 ml. of  $\text{H}_2\text{O}$ . The side arm contained 0.20 ml. of octanoate (0.001 M) or acetoacetate (0.005 M) and 0.20 ml. of  $\text{H}_2\text{O}$  or oxalacetate (0.01 M) or fumarate (0.01 M). Time, 18 minutes; temperature  $26^\circ$ .

Substrate	Oxalacetate	Fumarate	O <sub>2</sub> uptake	Change in acetoacetate	Formation of citrate
			<i>c mm.</i>	<i>c mm.</i>	<i>c mm.</i>
1. None	—	—	1	+1	2
2. Octanoate	—	—	75	+49	4
3. Acetoacetate	—	—	2	-2	1
4. None	+	—	92	+3	47
5. Acetoacetate	+	—	89	0	51
6. None	—	+	142	+4	24
7. Acetoacetate	—	+	149	-3	26

of acetoacetate may represent a major pathway of pyruvate metabolism (22, 23). This reaction has been proposed to occur by way of various hypothetical condensation products, such as parapyruvic acid (24),

acetopyruvic acid (22), etc. In the liver pyruvic acid also undergoes oxidation through the Krebs cycle (23).

The washed liver preparation used in this study is capable of oxidizing pyruvic acid and the various intermediates of the Krebs cycle. In the absence of oxalacetate or its precursors pyruvate is oxidized with the formation of acetoacetate, a reaction which was found to require adenine nucleotide for maximum activity. When this reaction was studied quantitatively, the data showed that the reaction can be expressed by the equation



This equation demands that the ratio of acetoacetate formed to oxygen used to pyruvate used (in molar quantities) is 1:1:2. The typical experi-

TABLE III

*Formation of Acetoacetate during Pyruvate Oxidation*

The main compartment of the Warburg vessel contained 0.60 ml. of enzyme, 0.20 ml. of  $\text{MgSO}_4$  (0.005 M), 0.20 ml. of sodium malonate (0.01 M), 0.20 ml. of phosphate buffer, pH 7.7 (0.007 M), 0.20 ml. of adenosine triphosphate (0.0012 M), and 0.20 ml. of  $\text{H}_2\text{O}$ . The side arm contained 0.40 ml. of sodium pyruvate (0.015 M) or  $\text{H}_2\text{O}$ . Time, 53 minutes; temperature  $31^\circ$ .

Pyruvate	$\text{O}_2$ uptake	Acetoacetate formation	Pyruvate removed	Ratio, acetoacetate formed to $\text{O}_2$ uptake to pyruvate removed
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	
—	0	2		
+	86	86	164	1.00:1.02:1.95
—	0	0		
+	72	70	140	1.00:1.03:2.00
Calculated, equation (2)				1.00:1.00:2.00

ments in Table III show very nearly a quantitative correspondence to this ratio.

It is evident that the washed enzyme suspension is capable of converting either fatty acids or pyruvate into acetoacetate quantitatively, according to equations (1) and (2).

*Formation of Citrate from Pyruvate*—Preliminary experiments showed that the liver suspension was capable of oxidizing pyruvic acid further than equation (2) indicated, but only in the presence of fumarate, malate, or oxalacetate, indicating the existence of two possible metabolic pathways for pyruvate oxidation in these preparations. In order to demonstrate these two pathways the oxidation of pyruvate by the malonate-inhibited liver suspension was studied in the presence and absence of simultaneous

fumarate oxidation. In the absence of fumarate, pyruvate was quantitatively oxidized to acetoacetate, as was shown in the previous section. When fumarate was oxidized simultaneously with pyruvate, the yield of acetoacetate dropped considerably and there was an extra accumulation of citrate (see Table IV). In the latter experiment there was evidently an accumulation of those intermediates of the Krebs tricarboxylic acid cycle preceding the point of malonate inhibition; namely, succinic dehydrogenase. Although citric acid is not on the main pathway of the Krebs cycle (25), it is in equilibrium with *cis*-aconitate and isocitrate, which are probably true intermediates in the cycle. The accumulation of extra citrate during pyruvate oxidation indicates that pyruvate can undergo oxidation via the Krebs cycle in the presence of fumarate (a precursor of oxalacetate). Pyruvate may therefore be oxidized through two pathways by these liver

TABLE IV  
*Acetoacetate and Citrate Formation from Pyruvate*

The main compartment contained 1.20 ml of enzyme, 0.40 ml of  $\text{MgSO}_4$  (0.005 M), 0.40 ml of phosphate buffer, pH 7.7 (0.008 M), 0.40 ml of sodium malonate (0.01 M), 0.40 ml of adenosine triphosphate (0.0014 M), and 0.40 ml of  $\text{H}_2\text{O}$ . Side arm, 0.20 ml of  $\text{H}_2\text{O}$  or pyruvate (0.015 M) and 0.20 ml of  $\text{H}_2\text{O}$  or fumarate (0.003 M). Time, 28 minutes, temperature 25°

Pyruvate	Fumarate	Pyruvate removal	$\text{O}_2$ uptake	Acetoacetate formed	Citrate formed
		<i>c mm</i>	<i>c mm</i>	<i>c mm</i>	<i>c mm</i>
—	—		2	2	3
+	—	310	162	157	6
—	+		93	7	45
+	+	337	274	54	105

suspensions, one leading to the formation of acetoacetate and the other through the events of the Krebs cycle. The course of the oxidation is determined by the supply of oxalacetate or its precursors. Acetoacetate is not a common intermediate in these two pathways, since it is not capable of forming extra citrate in the presence of fumarate or oxalacetate (Table II).

*Effect of Fumarate on Course of Fatty Acid Oxidation*—In a previous study it was shown that fatty acid oxidation requires "activation" by ATP or oxidations in the Krebs cycle (11). In that study routine acetoacetate determinations were made to demonstrate that extra oxygen uptake in the presence of fatty acid reflected actual oxidation of the fatty acid. On further study it was found that the ratio of acetoacetate formed to extra oxygen taken up differed widely, depending on the manner in which the oxidation of octanoate was "activated." When fatty acid oxidation was

activated by ATP or coupled to the oxidation of  $\alpha$ -ketoglutarate to succinate (in a malonate-inhibited system), the ratio of acetoacetate formed to extra oxygen taken up was approximately 0.60 to 0.65 (or nearly a quantitative correspondence to the stoichiometry of equation (1)). When  $\alpha$ -ketoglutarate was oxidized to succinate in the presence of malonate, the further oxidation of succinate to fumarate was obviously prevented. However, when octanoate oxidation was activated by fumarate oxidation in the presence of malonate, the ratio of acetoacetate formed to extra oxygen taken up dropped to as low as 0.20. Since acetoacetate was *not* attacked by the liver suspension (Table II), these results indicated that the production of acetoacetate was affected by the presence of fumarate or its oxidation products. This effect of fumarate in lowering the yield of acetoacetate from octanoate resembles the effect of fumarate in lowering the yield of

TABLE V  
*Acetoacetate and Citrate Formation from Octanoate*

The main compartment contained 1.20 ml. of enzyme, 0.40 ml. of  $\text{MgSO}_4$  (0.005 M), 0.40 ml. of phosphate buffer, pH 7.5 (0.004 M), 0.40 ml. of sodium malonate (0.01 M), 0.40 ml. of adenosine triphosphate (0.0018 M), and 0.40 ml. of  $\text{H}_2\text{O}$ . The side arm contained 0.20 ml. of  $\text{H}_2\text{O}$  or octanoate (0.001 M), and 0.20 ml. of  $\text{H}_2\text{O}$  or fumarate (0.007 M). Time, 29 minutes; temperature, 25°.

Octanoate	Fumarate	$\text{O}_2$ uptake	Acetoacetate formation	Citrate formation
		<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
—	—	2	0	0
+	—	201	130	2
—	+	118	2	46
+	+	327	57	82

acetoacetate from pyruvate demonstrated in the preceding section. This resemblance suggested the participation of fragments from the fatty acid in the Krebs cycle. To test this question acetoacetate and citrate formation from octanoate in the malonate-inhibited liver suspension were determined in the presence and absence of simultaneous fumarate oxidation. In Table V is shown a typical experiment.

It can be seen that, when octanoate was oxidized in the presence of fumarate, extra citrate was formed and the yield of acetoacetate was diminished. Since the extra oxygen uptake in the presence of octanoate was approximately the same with or without added fumarate, very likely the same amount of fatty acid underwent oxidation in the two situations. The implication of this experiment is clear; fatty acid oxidation may proceed to acetoacetate or through the Krebs cycle, depending on the supply of oxalacetate, exactly as pyruvate oxidation may undergo two metabolic

pathways in these preparations. Furthermore, acetoacetate does *not* form citrate in the presence of oxalacetate or fumarate (Table II), indicating that if oxalacetate acts by condensing with some reactive fragment to form citrate (or its precursors) it must react with some precursor of acetoacetate.

Similar experiments showed that the yield of citrate depends on the concentration of fumarate. Also, it was found that oxalacetate can be substituted for fumarate in these experiments. However, oxalacetate undergoes rapid decomposition into pyruvate and  $\text{CO}_2$ , introducing the complication of pyruvate oxidation. Fumarate oxidation was found to yield a more constant supply of the condensing intermediate with a reduction of this secondary complication to a minimum.

*Carbon Balance during Octanoate Oxidation*—The amount of citrate formed from octanoate indicated that the carbon of the fatty acid diverted from acetoacetate formation might be accounted for in the accumulation of intermediates before the malonate block. To determine this on a more nearly quantitative basis the accumulation of acetoacetate, citrate,  $\alpha$ -ketoglutarate, and succinate was measured during octanoate oxidation in the presence of fumarate. The other intermediates likely to accumulate under these conditions are *cis*-aconitate, isocitrate, and oxalosuccinate (26). These are not readily determined under these experimental conditions but, since *cis*-aconitate and isocitrate are in equilibrium with citrate (aconitase), their total accumulation can be calculated, since Martius and Leonhardt (27) have shown that at equilibrium isocitrate and *cis*-aconitate form 10.8 per cent of the total concentration of the reactants present.

In Table VI is presented a carbon balance sheet for the oxidation of octanoate, with and without simultaneous fumarate oxidation. Octanoate was not determined but it was assumed that the same amount of fatty acid disappeared in the two situations, since the extra  $\text{O}_2$  uptake in the presence of fatty acid indicated that this approximation was justified. It should be mentioned that the oxygen uptakes due to fumarate and fatty acid were not always additive in different preparations when both substrates were present, indicating in such cases a competition for hydrogen acceptors. Obviously, without actual determinations of octanoate disappearance it cannot be assumed that the same amount of octanoate is oxidized in the presence and absence of simultaneous fumarate oxidation unless the separate oxygen uptakes are additive. Therefore the analytical data reported were obtained from a selected experiment in which this additivity was evident during the course of the oxidation. It would of course be desirable to have, in addition to the data given, estimations of octanoate disappearance. The determination of octanoate by distillation and titration in the amounts used in these experiments was in our hands subject to considerable errors, since the

amount of octanoate substrate added per vessel is equivalent to but 0.80 ml. of 0.01 N NaOH. The determinations of succinate and citrate also

TABLE VI

*Carbon Balance during Octanoate Oxidation in Presence and Absence of Fumarate*

The Warburg vessels contained 2.40 ml. of enzyme, 0.80 ml. of  $\text{MgSO}_4$  (0.005 M), 0.80 ml. of phosphate buffer (0.008 M), 0.80 ml. of sodium malonate (0.01 M), 0.80 ml. of adenosine triphosphate (0.0019 M), and 1.40 ml. of  $\text{H}_2\text{O}$ . The side arm contained 0.40 ml. of  $\text{H}_2\text{O}$  or octanoate (0.001 M) and 0.60 ml. of  $\text{H}_2\text{O}$  or fumarate (0.009 M). Time, 34 minutes; temperature  $31^\circ$ .

Octanoate	Fumarate	$\text{O}_2$ uptake	Acetoacetate formed	Citrate formed	$\alpha$ -Ketoglutarate + succinate formed
		c.mm.	c.mm.	c.mm.	c.mm.
—	—	2	2	3	0
+	—	398	247	11	5
—	+	210	7	89	86
+	+	640	139	173	194

*Carbon Balance Sheet\**

		Carbon
		c.mm.
No fumarate	Octanoate C disappeared (calculated from $\text{O}_2$ uptake, equation (1))	$\frac{1}{2} \times 396 = 1056$
	C recovered	
	As acetoacetate	$4 \times 245 = 980$
	“ citrate	$2 \times 8 = 16$
	“ $\alpha$ -ketoglutarate + succinate	$2 \times 5 = 10$
Total recovered .....		1006 or 95%
With fumarate	Octanoate C disappeared (assumed equal from extra $\text{O}_2$ uptake)	= 1056
	C recovered	
	As acetoacetate	$4 \times 132 = 528$
	“ citrate	$2 \times 84 = 168$
	“ $\alpha$ -ketoglutarate + succinate	$2 \times 108 = 216$
	“ isocitrate + cis-aconitate (calculated)	= 17
Total recovered .....		929 or 88%

\* 1 microatom of C = 22.4 c.mm. See the text for assumptions.

require relatively large aliquots of the reaction media. Complete analysis of the reactants involved would therefore require an experiment of unwieldy dimensions. The assumption that the same amount of octanoate wa<sup>3</sup>

oxidized in the two situations would, however, appear to be justified, as is evident from the data.

In these calculations it was also assumed that a single 2-carbon fragment (see "Discussion") from the octanoate went into the formation of each molecule of citrate, isocitrate, *cis*-aconitate,  $\alpha$ -ketoglutarate, and succinate, and that two such fragments went into the formation of each molecule of acetoacetate. Also the analytical values for the control experiment (fumarate alone) were subtracted from the data.

The recovery of octanoate carbon in the absence of fumarate was 95 per cent, nearly all of it in the form of acetoacetate. In the presence of fumarate 88 per cent of the carbon was accounted for. Somewhat over half of this amount was recovered as acetoacetate and the rest as tricarboxylic acids,  $\alpha$ -ketoglutarate, and succinate. In view of the assumptions involved in the calculations and the errors inherent in the analytical methods the recovery may be considered essentially complete. Two additional recovery experiments yielded somewhat different distributions of carbon with recoveries of 91 per cent and 108 per cent. These carbon recovery experiments indicate that the process of fatty acid oxidation in these enzyme preparations can be quantitatively described by the formation of acetoacetate and the participation of fragments derived from the fatty acids in the events of the Krebs tricarboxylic acid cycle.

Comparisons of the relative effectiveness of pyruvate and octanoate in causing citrate formation were made with the same sample of liver suspension in the presence of excess fumarate and malonate. Obviously such comparisons are only semiquantitative, since the accumulation of citrate under these conditions is the resultant of several complex reactions. However, in a total of nine experiments in which citrate formation from pyruvate and octanoate was measured under identical conditions, the formation of citrate from the two substrates was always of approximately the same magnitude, although the formation of citrate from octanoate was usually somewhat smaller than from pyruvate. These experiments indicate that potentially fatty acids may contribute as much carbon for oxidation through the Krebs cycle as does pyruvate.

*Oxidation of Odd Carbon Fatty Acids*—It has been known for some time that the odd carbon fatty acids are less ketogenic than their even carbon neighbors and that they are glycogenic in contrast to the even carbon acids, which do not form extra liver glycogen in fasted rats. It is also known (28) that, as the C chain length of the odd carbon acids is increased, the glycogenic effect per carbon atom of the fatty acid fed decreases. This has been interpreted to mean that fatty acids are oxidized from the carboxyl end of the chain forming 2-carbon ketogenic fragments until the terminal 2 or 3 carbons are reached. The terminal 2-carbon fragment is ketogenic,

whereas the terminal 3-carbon fragment, in the case of the odd carbon acid, may be oxidized to pyruvic acid, which is glycogenic. As the chain length is increased, the terminal glycogenic 3-carbon fragment of the odd carbon fatty acid exerts relatively less glycogenic action in contrast to the much larger number of ketogenic fragments.

In view of this essential difference in the metabolism of the odd and even carbon fatty acids, it was of interest to examine the oxidation of odd carbon fatty acids in the washed liver suspension used in this study. In Table VII are shown analytical data obtained in a comparison of the rates of oxygen uptake, acetoacetate formation, and citrate formation when heptanoate, octanoate, and nonanoate were used as substrates. It can be seen that the neighboring odd carbon acids were oxidized at rates

TABLE VII  
*Oxidation of Odd and Even Carbon Fatty Acids*

The main compartment of the Warburg vessel contained 1.40 ml. of enzyme, 0.40 ml. of  $\text{MgSO}_4$  (0.005 M), 0.40 ml. of phosphate buffer (0.004 M), 0.40 ml. of sodium malonate (0.01 M), 0.40 ml. of adenosine triphosphatè (0.0018 M), 0.30 ml. of  $\text{H}_2\text{O}$  or fatty acid (sodium salt) (0.001 M), and 0.2 ml. of  $\text{H}_2\text{O}$  or fumarate (0.0075 M). Time, 27 minutes; temperature  $31^\circ$ .

Substrate	Fumarate	$\text{O}_2$ uptake	Acetoacetate formed	Citrate formed
		<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
	—	0	1	2
Heptanoate	—	224	136	5
Octanoate	—	238	156	3
Nonanoate	—	197	118	3
	+	164	4	36
Heptanoate	+	401	72	69
Octanoate	+	406	82	64
Nonanoate	+	362	61	63

approximating the rate of octanoate oxidation and that acetoacetate formation likewise proceeded at about the same rate from the three substrates. The odd carbon acids were also capable of citrate formation during simultaneous fumarate oxidation. The data therefore indicate that odd and even carbon fatty acids are oxidized by essentially the same mechanism. These data and conclusions are not necessarily inconsistent with the facts and interpretations concerning odd carbon fatty acid metabolism outlined above. For instance, the 9-carbon fatty acid may form on oxidation three 2-carbon fragments, which condense to form acetoacetate. The terminal 3-carbon fragment is very likely oxidized to pyruvic acid, which in the intact animal is glycogenic but in these washed liver suspensions is quantitatively ketogenic in the absence of fumarate, as has been shown in this



paper. Therefore one would expect no essential difference in the yield of acetoacetate from 8- and 9-carbon acids in these preparations. The mechanism of oxidation of odd and even carbon acids therefore appears to be identical in these preparations, and the differences observed *in vivo* may be ascribed to a difference in the metabolic disposition of the terminal 2- or 3-carbon fragment of the fatty acid.

#### DISCUSSION

This quantitative study of the over-all process of fatty acid oxidation in rat liver suspensions, uncomplicated by other metabolic events, renders much more approachable the study of the finer details of the enzymatic mechanisms of fatty acid oxidation. It has not been possible in the past to set up a balance sheet for this process because of the obvious limitations of the surviving slice and perfusion techniques. No statement may be made from the results of this study concerning the existence or importance of other pathways of fatty acid oxidation which have been postulated in the past, such as  $\omega$  oxidation. From the high activity of these liver suspensions in catalyzing the reactions described it would appear that these reactions are of major importance in fatty acid oxidation in the intact liver.

Recent work, summarized and added to by Buchanan *et al.* (6), has already extended the significance of the Krebs tricarboxylic acid cycle, which was originally postulated to account for pyruvate oxidation in muscle, to include the oxidation of acetoacetate in some extrahepatic tissues. In view of the experiments reported in this paper the significance of the cycle must be extended further to include the oxidative degradation of fragments derived from the fatty acid molecule in the liver. The Krebs cycle therefore assumes a more generalized and broadened importance as a cyclic mechanism for the oxidation of specific metabolites derived from both carbohydrate and fat.

The quantitative formation of 2 molecules of acetoacetate during the oxidation of octanoate demonstrated in this paper provides an independent and more conclusive confirmation of the "multiple" formation of ketones from fatty acids which has been observed in surviving slices and in intact animals (7-9). The effect of the 4-carbon dicarboxylic acids in reducing the yield of acetoacetate from octanoate by causing fragments from the fatty acid to pass through the Krebs cycle instead must be included as one of the factors in the complex process known as antiketogenesis. This effect may account in part for the fact that multiple formation of ketones *in vivo* is readily demonstrable only in fasting or diabetic animals.

Buchanan *et al.* (6) have already pointed out that the participation of fragments derived from fatty acids in the Krebs cycle does not imply that these compounds must necessarily cause extra glycogen synthesis in

the fasted rat, since in the process of oxidation of tricarboxylic acid to oxalacetate (or pyruvate) the carbon atoms of these reactive fatty acid fragments are lost as  $\text{CO}_2$ , leaving no net increase of glycogenic carbon. However, participation of fragments derived from fatty acids in the Krebs cycle does provide a possible explanation for the incorporation of isotopic fatty acid carbon into glycogen (29), a process which does not necessarily proceed through fixation of metabolic carbon dioxide derived from fatty acid (30).

The mechanism of acetoacetate and citrate formation from pyruvate or fatty acids is a subject of particular interest at present. It is tempting to assume that there exists a 2-carbon fragment common to these reactions as well as fatty acid and cholesterol synthesis (31, 32). Krebs has recently considered the possibility that pyruvate is oxidized to a 2-carbon compound prior to condensation with oxalacetate (33), although, as he points out, proof of this mechanism is still lacking. Weinhouse *et al.* (34) have already demonstrated the participation of 2-carbon fragments derived from fatty acids in acetoacetate synthesis. The oxidation of acetoacetate in extrahepatic tissues through the Krebs cycle also very likely involves preliminary splitting of the acetoacetate into two 2-carbon fragments (5, 6). In this connection, the author has already shown that kidney and muscle suspensions form acetate from acetoacetate (35). Since acetoacetate itself is incapable of forming citrate in the rat liver suspensions used here, it is evident that some precursor of acetoacetate (very likely a 2-carbon fragment) is the actual intermediate.

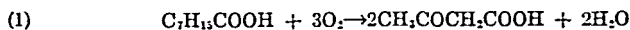
In view of these considerations, the most reasonable working hypothesis relating these metabolic events is that identical or, more likely, readily interconvertible 2-carbon fragments are involved in both fatty acid and pyruvate oxidation. Such a hypothesis has already been considered and elaborated in several recent publications (see (36)) and will not be developed in detail. In this connection, Bloch and Rittenberg (37) have shown that fatty acids and pyruvate differ in their ability to serve as sources of carbon for cholesterol synthesis and as sources of acetyl groups in the acetylation of foreign amines. Unpublished experiments of the author also indicate that not all of the fatty acid carbon is available for citrate synthesis in the liver suspensions used in this study. In view of these facts it is more probable that 2-carbon fragments from pyruvate and fatty acids are not actually identical but may be interconvertible under certain conditions.

An approach has been made toward the identification of 2-carbon compounds capable of forming acetoacetate and citrate. Acetate, glycolate, glyoxylate, oxalate, glycine, acetaldehyde, acetamide, ethanol, acetoin, and diacetyl do not appear to be intermediates in these reactions.

Acetyl phosphate, which has been almost universally suggested as the active intermediate in some of the metabolic reactions under discussion in view of Lipmann's excellent work on the identification of acetyl phosphate as an intermediate in certain bacterial reactions, is unable to form even traces of acetoacetate or citrate in the washed liver suspensions, even in the presence of ATP and simultaneous  $\alpha$ -ketoglutarate or fumarate oxidation. The rate of dephosphorylation of acetyl phosphate in these preparations is relatively slow and is not a factor in this failure.

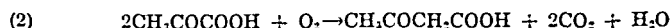
## SUMMARY

1. A washed rat liver enzyme suspension, in the presence of adenosine triphosphate, magnesium ions, and malonate, oxidizes octanoate completely to acetoacetate, according to the equation



There is no endogenous oxidative activity.

2. This enzyme suspension, in the presence of ATP, magnesium ions, and malonate, and in the absence of oxalacetate, oxidizes pyruvate quantitatively to acetoacetate, according to the equation



In the presence of oxalacetate, however, the yield of acetoacetate is diminished and extra citrate accumulates, evidence of the occurrence of the "Krebs condensation."

3. When fatty acids are oxidized by the enzyme in the presence of fumarate and malonate, the yield of acetoacetate is diminished and extra citrate,  $\alpha$ -ketoglutarate, and succinate accumulate in such amounts as to account *quantitatively* for the carbon of the fatty acid diverted from acetoacetate production. Acetoacetate does not form citrate in the presence of oxalacetate and ATP, indicating that the formation of citrate from fatty acid involves some *precursor* of acetoacetate.

4. Odd carbon fatty acids are oxidized and form acetoacetate and citrate at approximately the same rates as their even carbon neighbors.

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# THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDES\*

## VI. LIVER LECITHIN AS RELATED TO THE CHOLINE AND FAT CONTENT OF THE DIET

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Previous data in the literature point to a relationship between the proportion of fat in the diet and the formation of phospholipides in the liver. Thus, Artom (1) described a temporary rise of total phospholipides in the liver of dogs during the absorption of large amounts of fats. Sinclair (2) observed that the amounts of phospholipides were higher in the liver of rats maintained on a diet rich in fat than in control animals on a fat-free diet. In later investigations with the radioactive isotope of phosphorus ( $P^{32}$ ), Artom *et al.* (3) found that both the total radioactivity and the specific activity of the lipide P were increased in the liver of rats on a high fat diet.

The experiments reported in preceding papers of this series were designed to study the influence on tissue phospholipides of dietary factors other than fats. Accordingly, in order to obtain basal-like conditions, it seemed preferable to employ diets low in fat. On these low fat choline-deficient diets, a fall in total phospholipides and in lecithins of the liver was first observed (4). Under some conditions (*e.g.*, immediate choline supplementation in weanling rats (5, 6)), it was possible to prevent the decrease in lecithins to a large extent. In other experimental situations (*e.g.*, in 2 to 3 month-old rats with choline supplementation delayed (5, 7)), the drop in lecithins, as a rule, could not be prevented or reversed. In view of these observations and of the previous findings of Artom and Sinclair,<sup>1</sup> it now became of interest to investigate the effects of varying both the proportion of fat and of choline on the composition of the phospholipides of the liver.

### EXPERIMENTAL

Male albino rats, raised on our stock diet (Rockland Farms) to about 100 gm., were transferred to one of the experimental diets and maintained on

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<sup>1</sup> It should be noted that in these studies the diets probably contained adequate amounts of choline or choline precursors, and that total phospholipides only were determined in the liver.

the same diet for 19 days. The composition of the diets is recorded in Table I. Choline supplementation was initiated after 7 days and continued for 12 more days. At the end of the experiments, the animals were decapitated. Usually, the livers of the rats of the same group were pooled and minced; at least two samples of the pooled tissue were analyzed, as described previously (9). In other experiments, the analyses were carried out on samples from individual livers. Except for total fatty acids, all other determinations (P, choline, and dry weight) were made in duplicate.

### Results

The analytical data have been expressed on the basis of concentration, *e.g.* mg. per gm. of moist lipide-free tissue; the average values obtained in

TABLE I  
*Composition of Experimental Diets\**

The values are in per cent of dry weight.

Diet No.	Dextrin	Sucros <sup>2</sup>	Crisco	Cod liver oil
23	42	42		
20	39.5	39.5		5
24	39.5	39.5	5	
4	37	37	5	5
21	32	32	15	5
5	27	27	25	5
19	22	22	35	5

\* In addition to the above components, all experimental diets contained casein 10 per cent, a salt mixture (Osborne and Mendel (8)) 4 per cent, and a cellulosic material (Ruffex) 2 per cent. Pure B vitamins in amounts indicated previously (7) were incorporated in the daily rations of all animals. Moreover, the rats on Diets 23 and 24 received 2 drops of a concentrated preparation of A and D vitamins (oleum percomorphum), three times during the last 12 days of the experiments. In the experiments with choline supplementation, a 10 per cent solution of choline hydrochloride was added to the diets in amounts corresponding to 50 mg. per rat per day.

the various experimental groups are reported in Table II. For the purpose of comparison, the corresponding averages, previously obtained on the livers of animals on the stock diet (9, 7), are also included.

On the basis of these "normal" values, it is apparent that in the experiments without choline supplementation low levels for total and choline-containing phospholipides (but not for non-choline-containing phospholipides) have been uniformly obtained, irrespective of the proportion of fat in the diet.<sup>2</sup> As expected, in the absence of dietary choline, non-phospho-

<sup>2</sup> In the experiments without choline supplementation, the highest and lowest values for the choline-containing fraction were observed in the livers of the groups in Experiments 2 and 3; in these, two diets have been employed which contain the

TABLE II

*Lipide Composition of Rat Liver As Related to Proportion of Fats in Experimental Diets*

The rats were maintained on the experimental diets for 19 days. Choline supplementation was initiated after 7 days and continued for 12 days. Lipide values are calculated for 1 gm. of moist lipid-free tissue.

Experiment No.	No. of rats*	Diet No.	Per cent of fats in diet	Average daily food intake	Body weight		Liver weight	Total lipides	Phospholipides			Non-phospholipid fatty acids	Unsatifiable matter	
					Initial	Final			Total	Choline-containing	Non-choline-containing			
Experimental diets. No choline added														
				gm.	gm.	gm.	gm.	mg.	mg.	mg.	per cent of total phospholipides	mg.	mg.	mg.
1	2 (2)	23	0	15.7	106	147	7.70	268.9	23.6	10.4	44.1	13.2	218.3	5.2
2	3 (2)	20	5	12.6	106	141	6.83	207.5	26.3	15.1	57.4	11.2	157.0	8.5
3	2 (2)	24	5	10.7	104	120	6.36	116.7	18.6	7.6	40.9	11.0	81.2	8.8
4-6	9 (7)	4	10	12.1	107	149	6.98	164.9	23.6	12.4	52.5	11.2	120.6	8.6
7	3 (2)	21	20	8.9	109	119	6.32	171.9	24.8	14.4	58.1	10.4	129.0	5.2
8	4 (2)	5	30	7.6	110	133	7.13	312.3	21.6	11.1	51.4	10.5	256.0	9.1
9	4 (2)	19	40	5.8	104	105	6.46	248.7	25.0	13.0	52.0	12.0	193.2	11.2
Experimental diets. Choline added†														
10	2 (2)	23	0	16.8	105	158	6.96	59.1	24.9	14.2	57.0	10.7	26.3	5.3
11-12	8 (4)	20	5	10.2	105	128	5.57	42.9	24.3	16.2	66.7	8.1	13.2	4.1
13	2 (2)	24	5	14.5	107	144	6.20	48.6	22.3	15.4	69.0	6.9	18.8	5.6
14-16	9 (7)	4	10	10.1	102	137	5.73	37.3	23.2	13.5	58.2	9.7	9.0	4.2
17	4 (2)	21	20	10.5	103	130	5.26	56.9	28.2	20.0	70.9	8.2	20.9	5.7
18	4 (2)	5	30	8.2	102	129	5.76	72.5	21.1	17.9	84.8	3.2	41.3	6.0
19	4 (2)	19	40	5.6	102	113	6.11	80.0	28.6	22.0	77.0	6.6	40.6	6.7
Stock diet														
‡	24 (16)	§				122	5.56	44.5	31.5	19.5	61.9	12.0	8.6	3.6

\* The figures in parentheses indicate the number of samples analyzed.

† The amounts of choline hydrochloride actually ingested varied between 37 and 44 mg. (average 41 mg.) per rat per day.

‡ See (9, 6).

§ Rockland rat diet (complete), by Arcady Farms Milling Company, Chicago, Illinois.

same proportion of fat (5 per cent), however, with quite different characteristics. While these findings may be suggestive of a relationship between the nature of dietary fats and the lecithin level in the liver, the present data are obviously too scanty to justify any statement on this point.

lipide fatty acids were always elevated, although to a variable extent. In fact, even individual livers of animals in the same experimental group showed marked differences in their neutral fat content.

When the experimental diets were supplemented, after 7 days, with choline hydrochloride, choline-containing phospholipides were always more or less higher than in the corresponding groups on the same diets without choline. However, in the experiments in which diets with a low fat content

TABLE III  
*Comparison of Effects of Choline Supplementation on Liver Lipides of Rats on Low and High Fat Diets*

Average values calculated for	Per cent of fat in diet	Dietary supplement	No. of rats and analyses*	Phospholipides			Non- choline- contain- ing	Non-phos- pho- lipide fatty acids
				Total	Choline- containing			
				mg.	mg.	per cent of total phospho- lipides	mg.	mg.
1 gm. moist lipide-free tissue	0-10	Choline HCl	21 (15)	23.7	14.8	62.4	8.9	13
		None	16 (13)	23.5	12.1	51.5	11.4	135
		Difference		+0.2	+2.7	+10.9	-2.5	-122
	20-40	Choline HCl	12 (6)	26.0	20.0	76.9	6.0	34
		None	11 (6)	23.7	12.7	53.6	11.0	199
		Difference		+2.3	+7.3	+23.3	-5.0	-165
1 gm. dry lipid- free tissue	0-10	Choline HCl		97.1	60.6		36.5	53
		None		96.9	49.9		47.0	561
		Difference		+0.2	+10.7		-10.5	-508
	20-40	Choline HCl		113.1	87.0		26.1	138
		None		97.8	52.4		45.4	803
		Difference		+15.3	+34.6		-19.3	-665
Whole liver of 125 gm. rat	0-10	Choline HCl		123.1	77.0		46.1	63
		None		123.7	63.7		60.0	740
		Difference		-0.6	+13.3		-13.9	-672
	20-40	Choline HCl		142.1	109.1		33.0	190
		None		134.8	72.0		62.8	1097
		Difference		+7.3	+37.1		-29.8	-907

\* The figures in parentheses indicate the number of samples analyzed.

(up to 10 per cent) were employed, these differences are often slight, and most of the lecithin values obtained after choline administration fall in the higher range of the values found in the animals on experimental diets, not supplemented with choline. This finding is in agreement with our previous results on 2 month-old rats fed a diet containing 10 per cent fat (5, 7).

On the other hand, when the proportion of fat in the diet was raised to 20 per cent and above, the effects of choline supplementation on the lecithin



levels became more marked and values as high as in the livers of rats on the stock diet were obtained. The increase in the choline phospholipides was accompanied by a definite decrease in the non-choline phospholipide fraction. Consequently, in the liver of animals on high fat diets supplemented with choline the percentage of choline phospholipides in the total phospholipides is definitely elevated.

The determinations of non-phospholipide fatty acids show that the administered choline exerted its lipotropic effects in all animals, on both low and high fat diets.

In order to substantiate further the statements mentioned above, the analytical data have been calculated also on the basis of both the dry lipide-free tissue and the total amounts in the liver of a typical rat with a terminal weight of 125 gm. A comparison between the average values has been made in Table III from which it is also apparent that the same general conclusions may be drawn regardless of the method employed for expressing the results.

#### DISCUSSION

It appears that, under the conditions of the present experiments, the level of lecithins in the liver is dependent on the dietary supply of both choline and fat. When the experimental diets contain large proportions of fats and generous amounts of choline are also supplied, the lipide composition in the liver of 2 month-old rats becomes similar to that previously described in weanling rats on both high or low fat diets supplemented with choline (*i.e.*, high lecithin levels, non-choline phospholipides decreased, low content in neutral fats).

Total phospholipide values were increased only minimally, and not in all experiments. However, when differences in the experimental conditions are taken into account, our present findings may be considered to be in line with those of the studies mentioned previously (1-3), in which increases in the amount and in the rate of formation of total phospholipides have been described in the liver of animals fed high fat diets. It has been pointed out that these diets presumably contained adequate amounts of choline or choline precursors. On the basis of our present results, it may be assumed that the changes previously observed in the total phospholipides after ingestion of large amounts of fat were the expression of even more extensive changes in the lecithin fraction. This statement very probably applies also to the results of Perlman and Chaikoff (10), who found that the amount of newly formed phospholipides (as measured by the incorporation of radioactive phosphorus in the total phospholipides) is markedly increased in the liver of rats after the administration of a single dose of choline. In fact, in these experiments the animals were maintained on a

low protein diet containing as much as 40 per cent fat. Likewise, a high fat diet has been employed recently by Boxer and Stetten (11) who have shown, with the aid of isotopic nitrogen, that in the rat the incorporation of new choline into the liver lipides is accelerated during choline administration.<sup>3</sup>

#### SUMMARY

2 month-old rats were maintained for 19 days on experimental diets in which the percentage of fats was varied from 0 to 40 per cent. At this time, the livers of all the animals contained large amounts of neutral fats and low levels of total and choline-containing phospholipides.

When low fat diets were supplemented, after 7 days, with choline, the phospholipide values were only slightly affected. On the other hand, when choline was added to diets containing 20 per cent or more fats, the levels of choline phospholipides in the liver were as high as the corresponding values found in the liver of animals on a stock diet. This increase in the choline phospholipide fraction was accompanied by a decrease in the values for non-choline phospholipides.

It appears that, under the conditions of these experiments, the level of lecithin in the liver is dependent on the dietary supply of both choline and fat.

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<sup>3</sup> Since the completion of the present experiments, Entenman *et al.* (12) reported that in the liver of dogs injected with radioactive phosphorus the administration of choline increases the specific activity of the phosphorus in the choline-containing phospholipides, whereas in the non-choline-containing fraction the specific activity is definitely lowered.

# RELATION OF FASTING KETOSIS TO THE PROTEIN OF THE PRECEDING DIET

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The rapid onset of a marked fasting ketosis following low protein diets has been attributed by MacKay *et al.* (1, 2) to a lack of "stored" protein. They suggested that the catabolism of the "stored" protein after high protein diets, as measured by the urinary nitrogen excretion, supplied sufficient antiketogenic material (glucose) to reduce the need for metabolizing fats. Besides the increased excretion of nitrogen in the urine, the better maintenance of the blood sugar and the liver glycogen levels in fasting rats previously receiving more protein was cited as additional evidence for this mechanism.

Although there is considerable evidence (2, 3) that the protein of the preceding diet is related to the following fasting ketosis, it seemed desirable to obtain further data before concluding that the antiketogenic material from the protein catabolized during fasting, as measured by the nitrogen excreted, is sufficient to account for the low level of ketonemia following the higher protein diet. Also, a part of the small increase in nitrogen excreted by animals which had been on the 25 per cent protein diet, as compared with that of the animals which previously received a 5 per cent protein diet, might be due to either the well known lag in the excretion of the nitrogenous end-products of protein metabolism or to an excessive flushing out of these products from the body by the diuresis which was produced as a part of the experimental procedure (2).

The present report discusses the results of experiments designed to give additional information concerning two points in relation to fasting ketosis. First, is the extra nitrogen excreted by fasting animals previously on high protein diets (in comparison with those receiving low protein diets) an accurate measure of the quantity of antiketogenic material available from the "stored" protein catabolized in these animals? Secondly, does the "stored" protein catabolized during fasting supply sufficient antiketogenic material to prevent the development of a fasting ketonemia comparable to that observed in animals which have received low protein diets? In regard to the first point, this study indicates that the extra nitrogen excretion is a relatively accurate measure of the "stored" protein catabolized.

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Concerning the second, our results show that the administration of glucose, equivalent to the carbohydrate made available by catabolism of the extra protein (as calculated from the nitrogen excretion), did not produce in rats previously on a low protein diet the low level of fasting ketonemia observed in animals which had received high protein diets.

#### EXPERIMENTAL

In the first experiment white male rats averaging 200 gm. in weight were fed Diets 1 and 2 for 18 days. Diet 1 contained 5 per cent casein and 34 per cent each of starch and glucose;<sup>1</sup> Diet 2, 25 per cent casein and 24 per cent each of starch and glucose. Both diets contained 2 per cent Cellu flour, 5 per cent salt mixture (4), and 20 per cent lard. The diets were supplemented daily with 1 dried yeast tablet and 2 drops of cod liver oil. Beginning with the 19th day, all animals were fed Diet 1 for the next 3 days and then fasted for 2 days. The low protein diet was given for 3 days before the fast to the animals previously receiving 25 per cent protein to avoid any lag in the excretion of the nitrogenous end-products while fasting as a result of the earlier higher protein intake. Hence, the nitrogen excreted during the fast should be a truer measure of the protein catabolized. Diet 1 served as a maintenance diet for these animals, since all maintained their weight or gained slightly during the 3 day period. The daily food intake and weight changes were recorded. Daily urine collections were made under light mineral oil during the last 8 days of the study and the urine was analyzed for nitrogen. The adequate urine volumes previously found necessary for nitrogen excretion studies (2) were obtained by the intraperitoneal injections twice daily of 5 cc. of 0.9 per cent saline. The saline solution was administered for 6 days before the fast to prevent the flushing out of the end-products of protein metabolism during the fast, which might have occurred if the solution had been given only during the fasting period. Total blood ketone bodies were determined at the end of the 1st and 2nd days of fasting, as previously described (3).

In the second experiment, similar rats weighing approximately 225 gm. were fed Diet 3 for 21 days. This diet was similar to Diet 1 with the exception that the carbohydrate and fat contents were changed to 48 per cent glucose and 40 per cent lard. All of the animals received approximately the same amount of food, by limiting the food to that amount the majority would eat, in order that the body stores would be as nearly the same as possible when they were later divided into two groups. To determine more accurately the start of the fasting periods, the food cups were removed from the cages at 8 a.m. and returned at 9 a.m. On the day the fasting periods began, the cups were not returned to the cages and 9 a.m. was considered

<sup>1</sup> Generously supplied by the Corn Products Refining Company, New York.

the start of the fast. Intraperitoneal injections of 0.3 cc. per sq. dm. of body surface, calculated according to the formula of Rubner (5), of 0.9 per cent saline or 5 per cent glucose were given at the start of the fast and at the end of each 12 hour period of the 48 hour fast. This 15 mg. of glucose per unit of body surface given twice daily is equivalent to the anti-ketogenic material available from the metabolism of protein containing 8 mg. of nitrogen, assuming that the protein is 16 per cent nitrogen and that 58 per cent of the protein is available for antiketogenic action. The blood for ketone determinations was drawn just before the injections at the end of 24 and 48 hour fasting periods. The room temperature during both experiments was maintained between 25-27°. Apparent differences were tested for significance by the *t* method of Fisher (6), and only those having a *P* value of 0.01 or less were considered significant. The standard error of the mean is given with the tabulated data.

### Results

The nitrogen excretion of the animals on the higher protein diet promptly decreased when the animals were placed on the 5 per cent protein diet. As is shown in Table I, the average nitrogen excretion for this period was approximately the same as that of the 2 day fast, indicating that any lag in the excretion of nitrogen had been eliminated. However, there was still a significant difference in the nitrogen excreted during the fasting period by the two groups previously on the diets of differing protein content. An excess of approximately 8 mg. of nitrogen per unit of body surface per day was excreted by the animals previously on Diet 2. The shift from the high to a low protein diet for 3 days and the prolonged diuresis had not altered the significant difference in ketonemia after a 48 hour fast following the low and high protein diets.

Additional evidence regarding the nitrogen excretion during a 2 day fast was obtained by calculation with the data on the absolute weights of liver and other body proteins of animals from a previous experiment (7). The animals were the same strain and sex as those used in this study. Their weight, environment, and diets were quite comparable to those of the animals from which the nitrogen excretion data were obtained. The amount of protein available for catabolism was calculated according to Addis *et al.* (8), who reported losses of 20 per cent liver protein and 4 per cent of other body proteins in rats during a 2 day fast. In Table II are shown the differences between the liver and body proteins of the unfasted animals previously on low and high protein diets and the calculated losses during a 2 day fast. The excess protein calculated to be lost during the 2 day fast contained approximately 7 mg. of nitrogen per unit of body surface. This value is slightly less than the daily nitrogen excretion value of 8 mg. that we obtained experimentally.

The effect of injecting 15 mg. of glucose per sq. dm. of body surface twice daily on fasting ketonemia after the low protein diet is shown in Table III. This glucose, the equivalent of the antiketogenic material from the excess

TABLE I  
*Nitrogen Excretion and Fasting Ketonemia*

The rats were fed Diets 1 and 2 for the first 18 days. Then all were fed the 5 per cent protein diet during the 19th and 21st days and were fasted the 22nd and 23rd days. 24 hour urine collections were made for nitrogen determinations.

Diet No.	No. of rats	Initial weight	Change in weight	Protein intake per 100 gm. rat per day	Nitrogen excretion, sq. dm. per day			Fasting ketonemia	
					16th-18th days	19th-21st days	22nd-23rd days	22nd day	23rd day
		gm.	per cent	gm.	mg.	mg.	mg.	mg.	mg.
1	9	203	9.2	0.73	23.5 ±0.8	22.1 ±0.7	22.5 ±0.8	9.7 ±1.0	26.9 ±2.3
2	8	200	19.4	2.93*	66.3 ±2.4	32.6 ±0.7	30.9 ±1.0	10.4 ±0.7	18.8 ±1.7

\* During first 18 days; 2.62 gm. for 21 days.

TABLE II  
*Calculated Protein Available During 2 Day Fast*

The data including absolute weights of protein were obtained from a previous study (7). The excess available protein in rats on the 20 per cent protein diet over that of those fed the 5 per cent protein diet was calculated on the basis of a loss of 20 per cent of liver protein and 4 per cent of other body proteins during a 2 day fast (8).

No. of rats	Diet protein	Body surface	Absolute weights of protein		Protein per sq. dm.	
			Liver	Body	Liver	Body
	per cent	sq. dm.	gm.	gm.	gm.	gm.
8	5	2.43 ±0.05	1.050 ±0.048	22.62 ±0.79	0.432 ±0.014	9.309 ±0.297
9	20	2.87 ±0.03	1.281 ±0.036	29.82 ±0.73	0.446 ±0.016	10.390 ±0.215
Differences.....			0.231	7.20	0.014	1.081
Calculated protein available during fast.....					0.003	0.043
Nitrogen from available protein.....					0.000	0.007

protein catabolized during fasting following the high protein diet, was given to determine whether the effect on the following fasting ketonemia would be comparable to that of a preceding high protein intake. The level of

the blood ketone bodies appeared to be slightly lowered by even this small amount of glucose, but the difference between the ketone levels of these animals and the saline-injected controls was not statistically significant. Also, the ketonemia after the glucose injections was similar to the 26.9 mg. per cent value obtained in the first experiment after the 5 per cent protein diet when neither glucose nor saline was given during the fast.

The blood ketone values observed in this study were lower than some previously reported by this laboratory (3). These animals, which were obtained from a different commercial source, appeared to be more resistant to ketosis than those of the earlier studies. The higher values reported by others (2) may also be explained by the fact that their fasting periods began 15 hours after the removal of the food instead of at that time.

TABLE III

*Effect of Saline and Glucose Injections on Fasting Ketonemia*

In two groups, each containing twelve animals, the food intake per day was 7.1 gm. All the animals received a 5 per cent protein diet (No. 3) for 21 days. Injections of 0.3 cc. per sq. dm. of body surface of 0.9 per cent saline or 5 per cent glucose were given twice daily during the following 48 hour fast. This amount of glucose is that available from the metabolism of protein containing 8 mg. of nitrogen.

Body weight gm.	Change in weight per cent	Solution injected	Fasting ketonemia	
			24th hr. mg. per cent	48th hr. mg. per cent
223	-14.8	Saline	15.2 $\pm$ 1.0	28.5 $\pm$ 1.0
225	-14.2	Glucose	11.2 $\pm$ 0.6	25.6 $\pm$ 1.3

## DISCUSSION

An excess of 8 mg. of nitrogen per sq. dm. of body surface per day was excreted during the 2 day fast by the animal previously on the higher protein intake, even after a preceding protein maintenance diet for 3 days and 6 days of diuresis. It appears that this persisting difference in nitrogen excretion following the low and high protein diets cannot be ascribed to a lag in nitrogen excretion or to an excessive flushing out of additional nitrogenous end-products following the higher protein intake. Rather, the difference may be attributed to the catabolism of more protein in the fasting animal following the higher protein diets. This difference in excretion of 8 mg. of nitrogen agrees closely with the approximately 6 mg. value obtained by MacKay *et al.* (2) under similar experimental conditions with the exception of the prefasting maintenance diet and a shorter period of diuresis.

It appears significant that the calculated excess protein lost by the fast-

ing animals after the higher protein diet (Table II) should account for only slightly less nitrogen than the experimental values obtained in this study and in that of MacKay and coworkers. The evidence suggests that this amount of nitrogen (4 to 8 mg. per sq. dm. per day) is a measure of the excess protein catabolized by the fasting animal after higher protein intakes and that the higher value would represent the maximum amount of protein that might be catabolized to supply excess antiketogenic materials to these animals.

When the absolute amounts of liver and the remaining body proteins were converted to amounts per unit of body surface, the amount of liver protein was found to be approximately the same in the animals on both low and higher protein diets. A significantly greater amount of body protein occurred in the animals on the higher protein intake. This suggests that, under the conditions of this study, the body stores and not those of the liver supplied the additional protein metabolized in these animals with larger stores.

A supplement of glucose, equivalent to the excess protein catabolized during fasting after the higher protein diets, failed to produce the same low level of fasting ketonemia as did a previous higher protein intake. There appeared to be a slightly lower level of ketonemia in the fasting animals receiving this small amount of glucose, but the level was significantly higher than that of the animals previously on the higher protein intakes and was not significantly different from that of the animals after the low protein diets. Thus the available carbohydrate from the additional catabolized protein appears not to be the major factor responsible for the lower level of fasting ketosis after high protein diets, and this effect must at least in part be otherwise explained.

Evidence is being accumulated that suggests an increased rate of utilization of carbohydrate by the animals on the low protein diets, when fed and extending into the early fasting period. Other investigators (9, 10) have suggested alterations in the carbohydrate metabolism as a result of dietary changes. The rapid disappearance of liver glycogen during early fast (11) could be due to a more rapid utilization as well as a lack of replacement by glycconeogenesis. On a similar caloric intake, animals on the low protein diets usually lose weight or barely maintain it, while those on higher protein intakes make appreciable gains in weight. This suggests an increased rate of metabolism in these fed animals on low protein diets which would account for the early ketosis following the rapid loss of carbohydrate stores. This metabolic rate apparently decreases to below the normal levels after an overnight fast, since several investigators (12, 13) have shown a lower than normal basal rate in these animals. Further studies are in progress.



## SUMMARY

The increased nitrogen excretion during fasting after the higher protein diet was not eliminated by 3 days on a 5 per cent protein diet and 6 days of diuresis preceding the fast. Hence it appears not to be due to a nitrogen lag or an excessive flushing out of the metabolic end-products during the fast. A similar nitrogen value is accounted for by the extra protein excreted thus appears to be due to the additional protein catabolized in these animals.

A glucose supplement equivalent to the amount that would be available from the excess protein catabolized after the higher protein diet failed to produce the antiketogenic effect previously attributed to it. Thus the amount of protein available for catabolism during fasting does not appear to be the major factor responsible for the degree of fasting ketosis.

The greater fasting ketosis after a low protein intake may be related to an increased utilization of carbohydrate in these animals. This change in metabolism is suggested by the rapid disappearance of liver glycogen during the early fast and by body stores smaller than those of animals following high protein diets after similar caloric intakes.

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# A METHOD FOR THE RAPID DETERMINATION OF ALKALINE PHOSPHATASE WITH FIVE CUBIC MILLIMETERS OF SERUM

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The alkaline phosphatase of the serum increases early and markedly in rickets and returns completely to normal only after healing is complete. Because of this fact, serum phosphatase is the most satisfactory index now known for the detection of this deficiency. The phosphatase activity of serum is not strictly specific in this respect and has also proved clinically useful in a number of other pathological states; *e.g.*, Paget's disease, hyperparathyroidism, liver disease, etc.

In connection with nutritional studies on large groups of population, it became necessary to have a rapid method for the determination of this enzyme on small amounts of serum. By the use of a new substrate (*p*-nitrophenyl phosphate) a method has been devised which requires only 5 c.mm. of serum (0.005 ml.) and which permits 50 to 100 analyses to be made in 2 hours. The simplicity and speed of the method recommend it for macro- as well as microdeterminations and for either alkaline or acid phosphatase.

A number of methods have been described for the determination of the phosphatase content of serum and other biological materials, all of which depend upon the principle of measuring the rate of hydrolysis of various phosphate esters under specified conditions of temperature and pH. The two most widely used methods are those of Bodansky (1) and King and Armstrong (2) in which glycerol phosphate and phenyl phosphate respectively are employed as substrates. While these methods are satisfactory for many uses, they are rather time-consuming when large numbers of determinations are needed; furthermore, they require larger samples of serum than is convenient for the purpose of dietary surveys.

The substrate, *p*-nitrophenyl phosphate, was studied by King and Delory (3) and has been used for phosphatase estimations by Ohmori (4) and by Fujita (5). The compound is colorless, but upon splitting off the phosphate group, the yellow salt of *p*-nitrophenol is liberated (absorption maximum, 400 m $\mu$ ). Hence the substrate is itself an indicator of the amount of splitting and thus a measure of phosphatase activity. It is only necessary to incubate serum with the buffered reagent, stop the reaction

by dilution with alkali, and measure the amount of color developed. Since serum itself makes a small contribution to the color, the first colorimetric reading is followed by the addition of acid to the sample (converting the yellow sodium salt into colorless free nitrophenol) and a second colorimetric reading which furnishes a blank correction. This procedure is considerably simpler than those now in use. Furthermore, *p*-nitrophenyl phosphate is split by alkaline phosphatase 15 per cent faster than phenyl phosphate (3), 2 or 3 times more rapidly than glycerol phosphate, and 25 or 30 times faster than phenolphthalein phosphate (6). Because of this rapid splitting, and the high chromogenicity of the salts of *p*-nitrophenol, the reagent is well suited for adaptation to microprocedures.

### *Materials and Procedure*

#### *Reagents and Standards—*

*Reagent A.* Dissolve 7.50 gm. (0.1 mole) of glycine and 95 mg. (0.001 mole) of  $\text{MgCl}_2$  in 700 to 800 ml. of  $\text{H}_2\text{O}$ , add 0.085 mole (e.g. 85 ml., 1 *N*) of  $\text{NaOH}$ , and dilute to 1 liter.

*Reagent B.* Prepare 0.4 per cent disodium *p*-nitrophenyl phosphate (Eastman) in 0.001 *N*  $\text{HCl}$ . (At present the Eastman product contains about 50 per cent inert material; hence a double amount of this preparation should be used. If desired, the compound may be purified by recrystallization from hot 87 per cent alcohol.) If the pH is not 6.5 to 8.0, adjust with acid or base. To test for free nitrophenol, dilute 1 ml. with 10 ml. of 0.02 *N*  $\text{NaOH}$  and measure the light absorption at 415  $\text{m}\mu$ . If the extinction is greater than 0.08 (i.e. light transmission less than 83 per cent for a 1 cm. light path or 70 per cent for a 2 cm. light path), remove free phenol by extracting Reagent B two or three times with equal volumes of water-saturated butyl alcohol, and once with water-saturated ether, finally aerating off traces of ether. Store in the ice box. Reextract when Reagent B fails to pass the above test.

*Reagent C* (complete reagent). Mix equal parts of Reagents A and B. If necessary, adjust the pH to 10.3 and 10.4 with a little strong  $\text{NaOH}$  or  $\text{HCl}$ . Store in the refrigerator, or, better, store frozen. When 2 ml. plus 10 ml. of 0.02 *N*  $\text{NaOH}$  have an extinction (1 cm.) greater than 0.1, either discard or extract with butyl alcohol and ether as above and readjust the pH.

*Standards.* Prepare solutions containing 1, 2, 4, and 6  $\text{mM}$  per liter of *p*-nitrophenol (Eastman), mol. wt. 139.1.

*Apparatus—*For 5 c.mm. serum volumes, (a) 5 and 50 c.mm. Lang-Levy constriction pipettes (Fig. 1); (b) 6  $\times$  50 mm. serological tubes, Kimble No. 45060; (c) any spectrophotometer or photoelectric colorimeter adapted to 0.5 ml. volume measurements; e.g., Beckman spectrophotometer or

Junior Coleman spectrophotometer (model 6) (Adapters for the Coleman instrument may be obtained from Samuel Ash, 3044 Third Avenue, New York 56); (d) wire rack to hold 100 tubes; this may be made conveniently from  $\frac{3}{8}$  inch mesh wire screen.

*Principle and Use of Lang-Levy Constriction Pipettes*—These pipettes were originally described by Levy (7) working in the laboratory of Dr. Linderström-Lang. They are particularly useful for the easy, rapid, and precise measurement of volumes of 1 to 200 c.mm. (0.001 to 0.2 ml.), although larger pipettes are occasionally of value. With 10 to 200 c.mm. pipettes the percentage accuracy of delivery compares favorably with conventional pipettes delivering 2 to 10 ml.; i.e., approximately 0.1 per cent. Below 10 c.mm. the precision falls off somewhat, but even with 1 c.mm. volumes a precision of at least 1 per cent is obtained and may be much better with a well made pipette properly used. These pipettes are easy to construct for any one familiar with the rudiments of glass blowing,<sup>1</sup> or they may be obtained from the Arthur H. Thomas Company, Philadelphia.

Drawings of Lang-Levy pipettes are shown in Fig. 1. They are filled and emptied by the use of a small rubber tube such as is used with blood-diluting pipettes. The pipette is dipped not more than 1 or 2 mm. into

<sup>1</sup> The following directions are for making an ordinary 50 c.mm. constriction pipette. A 20 to 25 cm. length of either soft glass or Pyrex tubing, 4 or 5 mm. outer diameter, is heated in the middle and drawn down to a diameter of 1.5 to 2 mm. This furnishes material for two pipettes. 2 cm. from that point where the tube is narrowed (Fig. 1, a) the slender portion is further drawn down to a diameter of 0.5 to 0.8 mm. This narrowest portion is bent 1 cm. below the 2 mm. portion (b) at an angle of about 45° from a straight line and is cut off with a diamond point 3 or 4 mm. below the bend (c). The upper large end is fire-polished. A 0.1 or 0.2 ml. graduated pipette is partially filled with water and placed horizontally on the table, a rubber tube is attached to the new pipette, its tip is touched to the tip of the graduated pipette, and 50 c.mm. of water are sucked into the new pipette. A mark is made with the diamond point at the meniscus and just above this point the pipette is narrowed, for a distance of about 1 cm. or so (d-d), to an inner diameter of perhaps 1 to 1.5 mm., without thinning the wall. Once again 50 c.mm. of water are drawn in, and the meniscus should now fall in this narrow portion. If it does, a new mark is made with a diamond point and the actual constriction is made by heating just above this mark with as slender a flame as possible. Without pulling or pushing, the glass will thicken where the flame strikes it, and heating is continued until the bore is 0.1 to 0.2 mm. at its narrowed point (e). The constriction should be small enough to stop the meniscus from going by when moderate pressure is applied, but large enough so that undue pressure is not required to force the meniscus by. The opening at the tip (c) should be a trifle smaller than the upper constriction so that the pressure which pushes the meniscus past the upper constriction will not cause the pipette to deliver too rapidly. The delivery time for a 50 c.mm. pipette should be 2 to 5 seconds. With a little practice, pipettes within 1 to 2 per cent of the desired volume can easily be made. However, each pipette is subsequently calibrated by delivery of water into a weighing bottle containing moisture and weighing on a micro balance.

the liquid, and by sucking, liquid is pulled to just above the constriction. With gentle pressure the liquid level is blown down to the constriction, where surface tension stops the meniscus automatically. The pressure is not released until the tip of the pipette is removed from the remaining liquid. To deliver, the pressure is increased sufficiently to drive the meniscus past the constriction and this pressure is maintained until delivery is complete (2 to 5 seconds). With smaller pipettes the tip should be sufficiently constricted (by fire polishing) so that surface tension prevents air from following the liquid after it is delivered, since otherwise part of the liquid might be spattered. During delivery, the bend in the tip is used to keep the lower shaft of the pipette away from the wall of the tube into which the sample is being delivered. This is very important, since otherwise surface tension will cause the sample to run up the shaft of the pipette, and only part of the sample will be delivered into the tube, the rest clinging to the pipette. Similarly, the tip of the pipette must always touch a

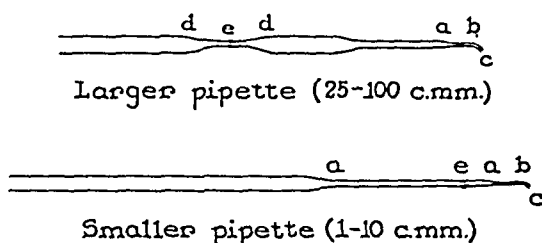


FIG. 1. Lang-Levy pipettes. See foot-note 1

surface during delivery, since if the tip is free in the air, much of the sample adheres to the outside of the pipette.

*Procedure with 5 C.mm. of Serum*—Serum samples (5 c.mm.) are transferred to the bottom of 6 × 50 mm. tubes in a wire rack. (A simple method for collecting small samples of serum has been previously described (8).) The rack is immersed in a shallow pan of ice water and to each tube are rapidly added 50 c.mm. of ice-cold Reagent C, with a constriction pipette. Each tube is mixed by tapping with the finger. Care is taken not to warm the tube by so doing.

The whole rack of tubes is now immersed in a water bath at 38° at a depth sufficient to cover the bottom half of the tubes. After exactly 30 minutes the rack is again placed in the pan of ice water and 0.5 ml. of 0.02 N NaOH is added to each tube with sufficient force to mix the sample. (A syringe pipette is very convenient for this purpose.) This addition stops the reaction and dilutes the samples which are now transferred to colorimeter tubes and read at 400 to 420 mμ =  $R_1$ .

After the initial reading, 2 to 4 c.mm. of concentrated HCl are added with a 0.1 ml. graduated pipette (drawn out tip) and a second reading,  $R_2$ , is made.

$R_1$  and  $R_2$  are converted into optical densities ( $-\log$  transmission or  $2 - \log$  per cent transmission) =  $D_1$  and  $D_2$ . Then  $D_1 - D_2 = D_c$ , the corrected density.

Standards and blanks are provided by treating 5 c.mm. volumes of the standards and of distilled water exactly as though they were serum samples. The corrected densities ( $D_c$ ) are used to construct a standard curve from which the serum values are calculated. Since sera and standards undergo the same dilution, it is unnecessary to take into account the exact volumes of the various pipettes. It is to be noted that  $D_c$  is the density corrected for possible residual absorption after acid addition but is not corrected for the  $D_c$  of the blank analysis. This second necessary correction is automatically provided by the standard curve. A "millimole unit" is defined as the phosphatase activity which will liberate 1 mM of nitrophenol per liter of serum per hour. Therefore, since the standard incubation time is only 30 minutes, the 1, 2, 4, and 6 mM standards are equivalent to sera with activities of 2, 4, 8, and 12 mM units. 1 such unit is approximately equal to 1.8 Bodansky units (see below). For adult sera, which have low phosphatase activity, the volumes of serum and reagent may be doubled without increasing the volume of alkali; this will nearly double the amount of color.

*Procedure with 20 C.mm. of Serum*—Volumes of serum, reagent, and 0.02 N NaOH are increased to 20 c.mm., 200 c.mm., and 2 ml., respectively. Otherwise the procedure is nearly identical with that described for 5 c.mm. of serum. The sample may be conveniently incubated directly in  $\frac{3}{8}$  inch photocolormeter tubes. 1 drop of 5 N HCl is added before the second reading.

*Procedure with 0.1 Ml. of Serum*—Use 0.1 ml. of serum, 1 ml. of reagent, and 20 ml. of 0.02 N NaOH. Because of the larger volumes, the tubes containing 1 ml. of reagent are placed in the water bath and allowed to come to temperature before the addition of serum. As each serum sample is added, the time is noted, and exactly 30 minutes later 20 ml. of NaOH are added.

#### DISCUSSION

Fig. 2 shows the differences in spectral absorption between *p*-nitrophenyl phosphate, *p*-nitrophenolate, and free *p*-nitrophenol. It is on these differences in absorption that the proposed method depends. Upon removing the phosphate group from *p*-nitrophenyl phosphate to form *p*-nitrophenolate, the absorption maximum is shifted from 310 to 400 m $\mu$  and

is nearly doubled in height. On converting the liberated nitrophenylate into free nitrophenol, by acidification, the absorption maximum is shifted back to  $318\text{ m}\mu$  and the absorption at  $400\text{ m}\mu$  is abolished. This latter reaction makes it possible to correct for the color contributed by the serum itself.

King and Delory (3) observed that the pH optimum of phosphatase with *p*-nitrophenyl phosphate as the substrate is more alkaline than with glycerol phosphate. The pH optimum with human serum was found to be 10.0 to 10.1, under the conditions described here (uncorrected glass electrode pH). Since alkaline phosphatase has a sharp pH optimum, devi-

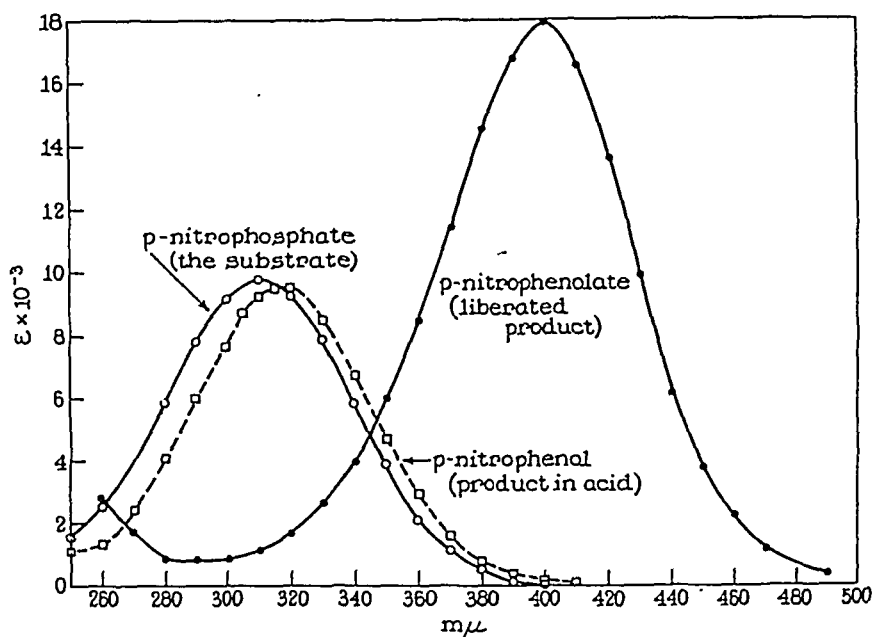


FIG. 2. Absorption curves of *p*-nitrophenol and *p*-nitrophenyl phosphate

ations of more than 0.1 pH unit will affect the readings significantly. For rat serum the optimum pH range is from 9.1 to 9.7. It is advisable to check the pH of the reagent against a standard buffer in the same pH range, such as 0.1 M sodium borate, pH 9.2. The reagent is well buffered; hence there is no danger of  $\text{CO}_2$  from the air affecting the activity during the incubation, but the pH of the reagent itself should be rechecked occasionally. Since the buffer capacity is large, it is permissible in the case of sera having very high phosphatase to reduce the volume of serum to one-half or one-quarter without changing the other volumes. The phosphatase reagent of Bodansky (1) is less well buffered and considerably more care



must be exercised with it to prevent pH changes from affecting phosphatase activity. The *p*-nitrophenyl phosphate reagent contains added magnesium, which, however, has less effect on the activity of the phosphatase than in the case of glycerol phosphate.

The degree of splitting in 30 minutes has been found to be proportional to the concentration of enzyme, but has not been found to be strictly linear with time for more than about 30 minutes. Therefore, it is desirable not to increase the period of incubation.

The blank correction for contribution of color from the serum is based on the finding that serum has very nearly the same absorption at 415 m $\mu$  in acid as in alkaline solution. Hemoglobin, if it is present as the result of

TABLE I  
*Comparative Results with Bodansky and Nitrophenyl Phosphate Procedures*

Serum No.	Bodansky units	<i>p</i> -Nitrophenyl phosphate units	Ratio, <i>p</i> -nitrophenyl phosphate to Bodansky units
1	8.36	4.68	1.79
2	8.80	4.98	1.77
3	10.7	5.68	1.88
4	8.30	4.81	1.73
5	9.30	4.76	1.95
6	9.27	4.76	1.95
7	5.60	3.27	1.71
8	7.42	4.22	1.76
9	7.14	4.32	1.66
10	8.36	4.99	1.67
11	4.48	2.41	1.86
12	6.54	3.70	1.77
Average			1.79
Standard deviation			0.10

hemolysis, absorbs considerably less light at 415 than at 400 m $\mu$ , which is one reason for preferring the longer wave-length. Hemoglobin does not absorb quite the same amount of light in acid and alkaline solution; hence excessive hemolysis should be avoided.

*Comparison with Other Methods*—In Table I are shown comparative results of analyses of twelve children's sera made by the Bodansky procedure (1) on 0.1 ml. of serum and by the method described here on 5 c.mm. of serum. It would appear that the Bodansky units (mg. of P liberated per 100 ml. of serum per hour) bear a ratio of 1.79 to the mm unit of the present procedure (mm of substrate hydrolyzed per liter of serum per hour). We have found the same ratio for adult sera, but the activities were so low that

accurate measurements by the Bodansky procedure were difficult to make, and hence a less regular correlation was found. In a personal communication, Dr. S. H. Jackson, Children's Hospital, Toronto, reported that the ratio of King-Armstrong units (mg. of phenol split from phenyl phosphate per 100 ml. of serum per hour) (2) to mm units is 7.3, with a variance of 8 per cent. With rat sera we have found in agreement with Fujita (5) that a much lower ratio is obtained. This observation deserves further investigation. The standard deviation of replicate determinations on the same serum by the proposed procedure with 5 c.mm. is 0.15 mm unit.

Huggins and Talalay (6) have recently introduced phenolphthalein phosphate as a phosphatase substrate. Like nitrophenyl phosphate it

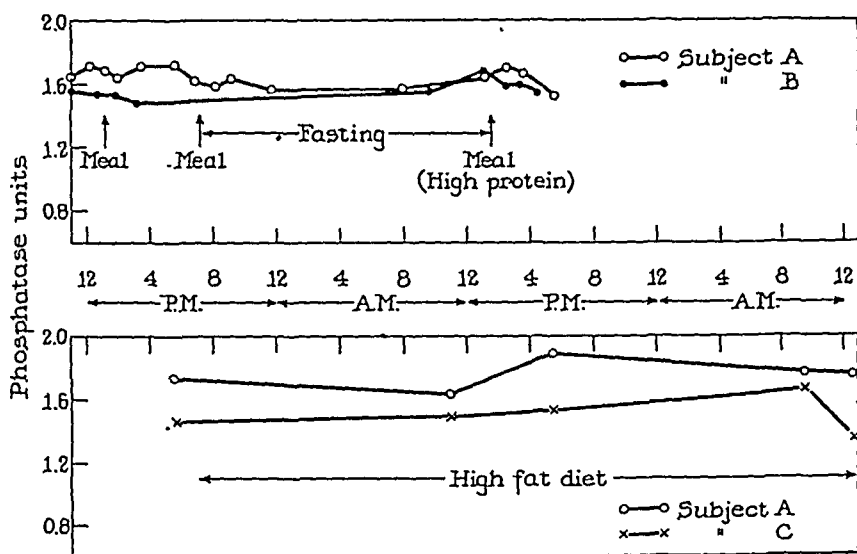


FIG. 3. Blood serum alkaline phosphatase under various dietary conditions

can function as its own indicator. It is, however, split only 3 or 4 per cent as fast as the latter and the color produced is stated not to be proportional to the enzyme concentration, presumably because there are two phosphate groups attached which may both have to be removed before color develops.

*Illustrative Data*—Fig. 3 gives the results of experiments to determine whether the alkaline phosphatase of human serum is influenced by the diet. Weil and Russell (9) observed that in the rat fasting for as little as 8 hours caused a marked decrease in serum alkaline phosphatase, confirming similar findings of Bodansky (10) for the dog. Gould (11) observed that a high fat diet produced an increase of 400 per cent in the alkaline phosphatase of rat serum. It is apparent (Fig. 3) that in the human subjects tested

there were no significant changes in the serum alkaline phosphatase attributable to an 18 hour fast, a high protein meal, or a 40 hour period of very high fat intake. The blood samples were all obtained by finger puncture. Duplicate 10 c.mm. serum samples were analyzed. It seems possible to conclude that for human phosphatase studies it is unnecessary to consider the immediate dietary history.

#### SUMMARY

A method is described for the determination of serum alkaline phosphatase, which permits analysis of 5 c.mm. (0.005 ml.) samples of serum at the rate of 50 to 100 per 2 hours. The simplicity and speed of the method also recommend it for macro- as well as microdeterminations, and for either alkaline or acid phosphatase.

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# A MICRODETERMINATION OF GLUTAMIC ACID AND ITS APPLICATION TO PROTEIN ANALYSIS\*

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The administration of glutamic acid to certain patients with petit mal epilepsy is followed by a marked decrease in the frequency of seizures and by increased mental acuity (2, 3). These surprising and unexplained observations suggest many metabolic investigations for which an accurate micromethod for the determination of glutamic acid in blood, tissue extracts, and body fluids is necessary. Such a procedure is described in this paper; the method is based on the fact that glutamic acid reacts with ninhydrin to yield  $\beta$ -formylpropionic acid, the 2,4-dinitrophenylhydrazone of which gives in alkaline solution a reddish brown color with a molecular extinction coefficient of approximately 28,000 at 420 m $\mu$ .

Since the  $\gamma$ -carboxyl of glutamic acid is almost inert if the ninhydrin reaction is carried out at pH 4.7, and quite inert at pH 2.5 (4), it may be expected that the formation of  $\beta$ -formylpropionic acid would be quantitative, and since available evidence indicated that glutamic acid is the only amino acid which forms an acidic aldehyde when treated with ninhydrin under the proper conditions, it was hoped that a specific separation by extraction of the acidic dinitrophenylhydrazone of  $\beta$ -formylpropionic acid from the neutral hydrazones derived from other amino acids could be easily accomplished. However, aspartic acid yielded a hydrazone of which amounts corresponding to as much as half of the aspartic acid present were extracted from an organic solvent into aqueous buffers of weak alkalinity. It was necessary, therefore, to separate glutamic acid from the interfering aspartic acid before the ninhydrin reaction was carried out. This was accomplished chromatographically with Wieland's "acid" aluminum oxide. Glutamic acid may be eluted with 0.5 N acetic acid, whereas aspartic acid remains on the column (5).

Ninhydrin forms a neutral dinitrophenylhydrazone, some of which might be carried mechanically into the final test solution. More serious would be interference from phenylglyoxal-*o*-carboxylic acid (6), which is derived from ninhydrin in alkaline solution and would form an acidic hy-

\* This work was aided by a grant from the Williams-Waterman Fund of the Research Corporation. A preliminary report has appeared elsewhere (1).

drazone. The removal of the excess of ninhydrin, made necessary by these considerations, was achieved through the formation of the ninhydrin-guanidine complex, which at the prevailing concentrations is soluble but is precipitated by lead acetate in alkaline solution.

After the introduction of the chromatographic separation, no interference from other amino acids was encountered, with the exception of cystine and cysteine which, after reaction with ninhydrin, form acidic hydrazones. However, the potential error from this source is relatively small and may be calculated and eliminated since the quantities of cystine and cysteine present may be determined by independent methods with high accuracy.

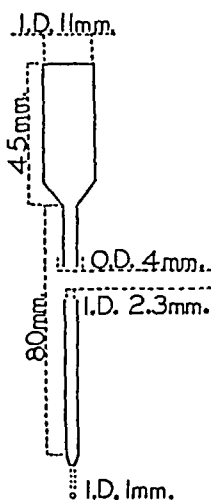


FIG. 1. Adsorption funnel

#### *Apparatus—*

*Spectrophotometer.* The Coleman Junior clinical spectrophotometer was used with No. 6302 cuvettes, which agreed with each other within  $\pm 0.72$  per cent.

*Centrifuge tubes.* 15 ml., graduated, and 25 ml., ungraduated.

*Glass tubes.* 7 mm. outer diameter, drawn out to 1 mm. tips.

*Adsorption funnels* (Fig. 1).

*Adapters* for packing the aluminum oxide in the adsorption funnels by centrifuging. Into cork plates, of 3 cm. diameter and 1 cm. thickness, are bored two holes corresponding to the diameter of the stem of the adsorption funnels. The adapter with the funnels in place is set in a 15 ml. centrifuge metal tube and the assembly is fitted into a 100 ml. centrifuge metal tube.

## Reagents—

*Ninhydrin.* Eastman, recrystallized once from boiling water with the addition of norit (yield 80 per cent).

*Acetic acid*, 0.5 N.

*14 per cent guanidine carbonate solution.*

*12 per cent neutral lead acetate (+3H<sub>2</sub>O) solution.*

*5 N NaOH.*

*0.1 per cent 2,4-dinitrophenylhydrazine solution in N HCl.* This reagent is kept in the refrigerator and renewed at monthly intervals.

*Capryl alcohol.* Treated with sodium and distilled.

*Absolute ethanol.*

*Borate buffer.* 20 gm. of sodium tetraborate and 5.3 gm. of sodium carbonate are dissolved in 1 liter of water and the pH is adjusted to 10.

*Aluminum oxide*, according to Brockmann (Merck).

*N HCl.*

*Standard solution of 2,4-dinitrophenylhydrazone of  $\beta$ -formylpropionic acid.* 2.5 gm. of ethyl formyl succinate are converted into  $\beta$ -formylpropionic acid by refluxing for 6 hours in 12.5 ml. of a 3 per cent solution of oxalic acid (7). The reaction mixture is cooled and added with shaking to 1 liter of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl. After several hours in the cold the hydrazone is filtered off (1.9 gm., 54 per cent yield) and recrystallized three times from 95 per cent ethanol (m.p. 202.5–203°, uncorrected).

A product prepared in this way gave the following analytical data.<sup>1</sup>

$C_{10}H_{15}O_2N_4$ (282.21).	Calculated.	C 42.6, H 3.6, N 19.9
	Found.	" 42.8, " 3.5, " 19.7

Titration with alcoholic KOH to the first color change gave an equivalent weight of 280.3.

From 50 to 60 mg. of the hydrazone (1 mg. = 0.521 mg. of glutamic acid) are dissolved in approximately 10 ml. of absolute ethanol with the addition of 1 ml. of pyridine. The hydrazone dissolves with difficulty and it is necessary to shake for about 30 minutes prior to dilution to 100 ml. with absolute ethanol. From this stock solution, which may be kept in the ice box for several days, a working standard is prepared by diluting 2 ml. to 100 ml. with borate buffer.

## Procedure

*Determination of Glutamic Acid in Mixtures with Other Amino Acids; Chromatographic Adsorption; Preparation of Column*—A suspension of 10 gm. of aluminum oxide in about 50 ml. of N HCl is stirred for approximately

<sup>1</sup> We are indebted to Mr. W. Saschek for the analyses.

1 minute, the acid is decanted, and the oxide is washed with distilled water by decantation eight to ten times until the washings are neutral to litmus. "Acid" aluminum oxide so prepared can be stored under distilled water.

The opening of the adsorption funnel is closed with a small amount of glass wool, gentle suction is applied, and about 6 cm. of the funnel stem, measured from the glass wool, are filled with the oxide. After filling the funnel with water it is centrifuged in the assembly described above for 30 minutes at 2000 R.P.M.

*Adsorption, Washing, and Elution*—The solution of glutamic and other amino acids is neutralized with dilute NaOH to pH 7, with the aid of a pH meter or with bromothymol blue as an internal indicator, and diluted with water to a volume such that 1 ml. contains not more than 25  $\gamma$  of glutamic acid. (Concentrations of sodium chloride up to molar do not interfere with the adsorption and elution of glutamic acid.) Two different dilutions of the solution are prepared and the three concentrations are each determined in duplicate. Thus, with two blank determinations, each sample analyzed requires a total of eight adsorption funnels.

For each determination, 2 ml. of the solution are filtered by gravity through a funnel. When the surface of the fluid reaches a point about 1 mm. above the oxide, 2 ml. of water are added. The filtrate and wash water are discarded. The elution is accomplished by passing two 2 ml. portions of 0.5 N acetic acid through the column into a 15 ml. graduated centrifuge tube.

*Reaction with Ninhydrin and Removal of Excess Ninhydrin*—To each of the eight tubes containing eluate,  $20 \pm 0.5$  mg. of ninhydrin are added and the tubes are immersed in a boiling water bath for exactly 10 minutes, after which they are transferred to an ice bath for 2 minutes. The following reagents are added in the order named with an interval of 5 minutes after each addition: 0.4 ml. of 14 per cent guanidine carbonate, 1 ml. of 12 per cent lead acetate, and 0.5 ml. of 5 N NaOH. The mixtures are diluted to 6 ml. with water, well stirred with rods, and centrifuged for 10 minutes. The supernatant solutions are decanted into small test-tubes.

*Formation and Extraction of Hydrazone*—During the centrifuging, 3 ml. portions of a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in N HCl are placed in eight 100 ml. test-tubes and cooled in an ice bath. Glass tubes drawn to a 1 mm. tip are placed in each of the tubes and 5 ml. portions of the supernatants are added. The solutions are mixed by passing air through the tubes for about 1 second. Exactly 10 minutes later, 10 ml. of capryl alcohol are added to each tube and the mixtures are agitated by a vigorous air stream for 1 minute. The tubes are taken from the ice bath, most of the aqueous phases are removed through the aeration tubes by



suction, the turbid capryl alcohol layers are clarified by centrifuging for 5 minutes in 15 ml. centrifuge tubes, 9 ml. aliquots of the capryl alcohol are pipetted into 25 ml. centrifuge tubes, and 6 ml. portions of borate buffer are added. The mixtures are vigorously agitated by a stream of air for 1 minute, and the layers are separated by centrifuging for 20 minutes.

*Development and Reading of Color*—A 5 ml. portion of one of the borate solutions is mixed with 2 ml. of absolute ethanol in a cuvette which is then immersed in a water bath at 25° for 5 minutes. The solution is then well mixed with 1 ml. of 5 N NaOH. Exactly 2 minutes after addition of the alkali, the color is measured in the spectrophotometer at 420 m $\mu$  against a blank containing 5 ml. of borate buffer, 2 ml. of absolute ethanol, and 1 ml. of 5 N NaOH.

*Preparation of Standard Curve and Calculation*—Different amounts, up to 5 ml., of the working hydrazone standard solution are diluted to 5 ml. with borate buffer in the cuvettes of the spectrophotometer, and the color is developed as described in the preceding section.

In the range of optical densities from 0.04 to 0.75 the standard curve approximates a straight line expressed by the formula  $G = 50D - 0.5$ , in which  $G$  is the glutamic acid (in micrograms) equivalent to the quantity of standard taken and  $D$  is optical density. The degree of deviation from this straight line relationship is indicated by the following values: 1.7  $\gamma$  ( $D = 0.04$ ), +0.3  $\gamma$ ; 9.7  $\gamma$  ( $D = 0.21$ ), +0.3  $\gamma$ ; 20  $\gamma$  ( $D = 0.41$ ), 0; 30  $\gamma$  ( $D = 0.60 \pm$ ), -0.3  $\gamma$ ; 38.1  $\gamma$  ( $D = 0.75$ ), -1.1  $\gamma$ .

The amount of glutamic acid present in the original sample is calculated from the formula, glutamic acid =  $1.6 \times F_c (A - B) - 0.12C$  where  $A$  is the quantity read from the standard,  $B$  the quantity corresponding to the blank, and  $C$  the amount (in micrograms) of cystine + cysteine. The factor 1.6 is derived from the fact that three aliquots (5/6, 9/10, 5/6) are taken during the procedure.  $F_c$  is a constant representing the retention of a portion of the glutamic acid on the aluminum oxide column and the distribution of the hydrazone between capryl alcohol and the aqueous acid and alkaline solutions. Since each of these factors depends on definite physical relationships, the value of  $F_c$  for the conditions of the determination can be established with precision (see below).

*Determination of Glutamic Acid in Absence of Interfering Substances*—When no interfering substances are present, the chromatographic adsorption is omitted and the reaction with ninhydrin is carried out in 4 ml. of 0.5 N acetic acid containing the glutamic acid. Two blanks containing 4 ml. of acetic acid are included in each set of determinations. In the calculation, instead of the constant  $F_c$ , a constant  $F_p$ , based on the distribution of the hydrazone between capryl alcohol and the aqueous solutions, is used.

## EXPERIMENTAL

*Determination of Constants  $F_p$  and  $F_e$* —In Experiments 1 and 2 (Table I), amounts of *dl*-glutamic acid varying from 5 to 50  $\gamma$  were carried through the procedure for determination in the absence of interfering substances; *i.e.*, with omission of chromatographic separation. In Experiments 3 and 4, blanks were carried through the same procedure, but hydrazone was added to the capryl alcohol in quantities corresponding to 10 to 30  $\gamma$  of glutamic acid per sample. The solutions were prepared by diluting the stock standard solution of hydrazone with capryl alcohol.

In interpreting the results account must be taken of a small variation of the constants, up to about  $\pm 3$  per cent of the mean, which we have not been able to control, in the results obtained with different preparations of reagents, particularly capryl alcohol. In practice we purify batches of capryl alcohol large enough to last about 2 months and simultaneously

TABLE I  
*Determination of  $F_p$  and  $F_e$*

Experi- ment No.	Type of determination	No. of deter- minations	Re- agents	Glutamic acid observed	Standard error	$F_p$	$F_e$
				<i>per cent</i>	<i>per cent</i>		
1	Glutamic acid in pure	24	A	83.4	$\pm 0.5$	1.20	
2	solution	41	B	85.9	$\pm 0.3$	1.16	
3	Recovery of hydrazone	6	A	83.4		1.20	
4		12	B	86.3	$\pm 0.6$	1.16	
5	Glutamic acid after chro-	76	A	71.6	$\pm 0.3$		1.40
6	matographic separation	19	B	73.7	$\pm 0.4$		1.36

all reagents, except the hydrazine solution, are renewed. The constants are determined in two sets of experiments with each new set of reagents and rechecked by glutamic acid determinations about every 10 days.

Experiments 1 and 3 were carried out with one set of reagents (A) and Experiments 2 and 4 with another (Reagent B). It will be seen that the recovery of glutamic acid and of hydrazone equivalent to glutamic acid was identical for each pair of experiments. This result shows that the loss of glutamic acid in the steps of the method after chromatography is accounted for entirely by the distribution factors of the hydrazone between capryl alcohol and the acid and alkaline aqueous solutions. The conversion of the amino acid into the corresponding aldehyde and the formation of the hydrazone are quantitative.

It would be possible to increase the yield by repeated extractions of the hydrazone with the alkaline buffer solution from capryl alcohol, but this

would complicate the procedure unnecessarily. As is shown by the small standard errors (Table I), the recovery, and  $F_p$ , which expresses it under the conditions of the method, is quite constant, as would be expected since the magnitude of the loss depends on definite physical properties of the solutions employed.

In Experiments 5 and 6, carried out with different sets of reagents amounts of *dl*-glutamic acid varying from 5 to 50  $\gamma$  were carried through the entire procedure in the presence of other amino acids, not including cystine and cysteine. The over-all loss represents that just discussed plus the loss which occurs during chromatography. The latter appears to result mainly from insufficient elution rather than from incomplete adsorption. In experiments in which a column of aluminum oxide of the same length but of larger diameter was used, the recovery of glutamic acid dropped considerably. The increased quantity of oxide would be expected to favor adsorption and to lessen the efficiency of elution. It is probable that the recovery could be increased by further elution, but the larger volume of eluate would complicate the procedure and would offer no advantage, since under the conditions described the loss is constant. The sets of reagents used in Experiments 5 and 6 were the same as in Experiments 1 and 2, respectively. When the average loss occurring in the chromatographic separation is calculated from these pairs of experiments, it is found to be exactly the same (14 per cent) in both experiments. The small difference in the  $F_c$  constants results from the effect of reagent variation in the recovery of the hydrazone.

*Determination of Glutamic Acid in Presence of Other Amino Acids and of Glutamine*—The introduction of the chromatographic separation became necessary when it was found that aspartic acid produced a hydrazone which appeared in the final borate solution. The efficiency of this procedure in eliminating interference by aspartic acid is demonstrated in Table II, which also includes data obtained in experiments in which various amino acids were added in the proportions shown. Interference was observed only by the group of acids including cystine. In fifteen experiments in which the molar ratio of cystine or cysteine to glutamic acid was varied from 6 to 0.1, it was found that  $12 \pm 2.6$  (standard error) per cent of cystine or cysteine, read as glutamic acid from the standard curve, appeared in the final borate solution. In one experiment without chromatographic adsorption, only about 16 per cent of the cystine present was recovered, a result which indicates that much cystine is adsorbed on the column and eluted by acetic acid.

As glutamic acid usually preponderates greatly over the two sulfur amino acids, a considerable error in determining the cystine and cysteine correction would represent only a small error in the glutamic acid value.

Attempts were made to remove cystine from mixtures with glutamic acid by the use of cuprous oxide, but large losses of glutamic acid occurred, probably referable to the large excess of cuprous oxide. Preliminary tests indicated that cysteic acid does not interfere in the determination, but

TABLE II  
*Interference of Amino Acids in Determination of Glutamic Acid*

Amino acids added	Glutamic acid to amino acid	Glutamic acid in sample*	Found	Deviation from glutamic acid in sample
	<i>molar ratio</i>	$\gamma$	$\gamma$	<i>per cent</i>
None		15.3	15.8	+3
Aspartic acid	1:1.5	15.3	15.3	0
	1:3	7.6	7.6	0
None		30.3	30.8	+2
Aspartic acid	1:0.85	30.3	30.8	+2
	1:1.7	15.2	15.4	+2
None		29.4	29.4	0
Tyrosine†	1:1.5	29.4	29.7	+1
None		15.7	15.2	-3
Serine	1:3	15.7	15.7	0
None		15.7	15.2	-3
Histidine†	1:3	15.7	15.6	-1
Hydroxyproline†	1:3	14.7	15.4	+5
Proline†	1:3	14.7	15.1	+3
Tryptophane†	1:3	14.7	15.0	+1
None		30.5	30.5	0
Valine, leucine, threonine, isoleucine	1:1.5	30.5	30.5	0
	1:3	15.3	15.5	+1
None		14.9	14.6	-2
Glycine, alanine, arginine†	1:3	14.9	14.6	-2
	1:3	14.9	14.2	-5
None		29.6	29.8	+1
Methionine, phenylalanine, tyrosine,† lysine, cystine	1:3	29.6	41.0	+38
	1:6	14.8	26.9	+82
Cystine	1:0.75	20.5	21.9	+7
Glutamine††	1:12.5	15.0	16.6	+11

\* *dl*-Glutamic acid hydrochloride (found, Cl 19.38; calculated, 19.32).

† Naturally occurring isomer.

†† We are indebted to Dr. H. B. Vickery for a sample of glutamine.

this approach had to be abandoned because the recovery of glutamic acid was decreased after oxidation of cystine to cysteic acid by bromine.

Of particular interest is the determination of glutamic acid in the presence of glutamine. The last experiment of Table II shows that 0.9 per cent of the glutamine present was determined as glutamic acid in the procedure. In experiments in which glutamine alone in amounts up to 50  $\gamma$  was tested,

approximately 1 per cent was recovered as glutamic acid. The negligible interference of glutamine makes the determination of glutamic acid possible in the presence of a rather large excess of the amide (8).

**Stability of Hydrazone in Acid and Alkaline Solutions**—Of a solution of approximately 30  $\gamma$  of hydrazone per ml. in alcohol-pyridine, 1 ml. portions were added to 4 ml. portions of 1.25, 0.125, and 0.0125  $N$  HCl in spectrophotometer cuvettes which were kept at 0° or at 25° for different time intervals. The samples were read exactly 2 minutes after the addition of 1 ml. of 5  $N$  NaOH (10  $N$  in the experiment with 1.25  $N$  HCl). The results, which are presented in Fig. 2, show that under the conditions of the method (10 minutes at pH about 1.3, determined with the glass electrode, and 0°) the destruction does not exceed 4 per cent.

In a similar series of experiments, 1 ml. portions of the hydrazone solution were added to 4 ml. portions of 10 per cent sodium carbonate solution

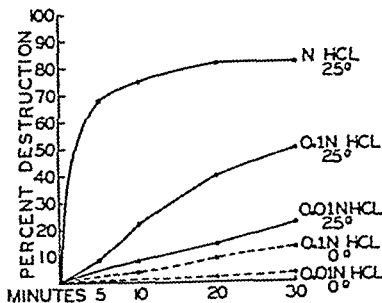


Fig. 2. Destruction of 2,4-dinitrophenylhydrazone of  $\beta$ -formylpropionic acid in  $N$ , 0.1  $N$ , and 0.01  $N$  HCl at 0° and 25°.

in cuvettes. After different intervals of time at 25°, 1 ml. of 5  $N$  NaOH was added and the color was read exactly 2 minutes later. In other experiments 1 ml. of 5  $N$  NaOH was added to each of a number of cuvettes containing approximately 30  $\gamma$  of hydrazone in 4 ml. of borate buffer. The samples were kept at either 25° or 40° and read at different time intervals after the addition of the sodium hydroxide solution. The reading at 2 minutes was taken as a base-line and the values plotted in Fig. 3 represent percentage decreases from this point. The results (Fig. 3) show a rapid destruction of the hydrazone in strongly alkaline solutions and a slower rate of decrease in carbonate solution. There was no detectable change during 30 minutes in borate buffer.

The instability of the hydrazone in the strongly alkaline solution which is required for the development of color necessitates rigid time and temperature control. A 2 minute interval, after addition of the alkali, and a ter-

perature of 25° were selected as most convenient for a routine determination.

*Determination of Glutamic Acid in Proteins*—Five proteins in amounts of 7 to 15 mg. were hydrolyzed with 1 ml. of constant boiling hydrochloric acid in sealed tubes at 120–125° for 24 to 28 hours (Table III). When cold, the content of each tube was neutralized with dilute sodium hydroxide solution and diluted to 100 ml. 1 or 2 ml. portions were taken for the analysis. Each protein was subjected to two separate sets of analyses with six determinations and two blanks in each set. When less than six values are reported (Table III), those missing were lost by breakage of centrifuge tubes.

For the casein we are indebted to Dr. M. Dunn. It is the same sample analyzed by Dunn and his collaborators (9) with the aid of *Lactobacillus*

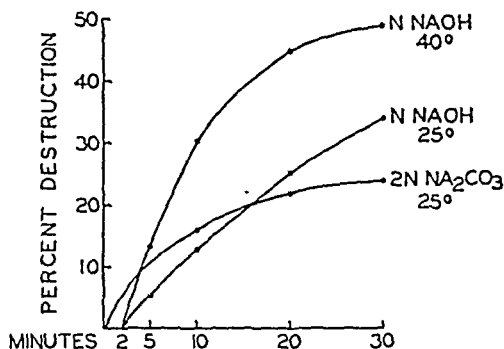


FIG. 3. Destruction of 2,4-dinitrophenylhydrazone of  $\beta$ -formylpropionic acid in N NaOH at 25° and 40° and in 2 N Na<sub>2</sub>CO<sub>3</sub>.

*arabinosus*. The lactoglobulin and egg albumin, for which we are indebted to Dr. R. K. Cannan, were prepared in the same way as the samples analyzed by Cannan (11). The bovine and human serum albumin were supplied through the courtesy of Dr. H. B. Vickery and were produced at the Department of Physical Chemistry, Harvard Medical School, under contract with the Committee on Medical Research of the Office of Scientific Research and Development.

The values (Table III) obtained in our analyses agree closely with those found with the isotope dilution method, on  $\beta$ -lactoglobulin (12) and on bovine and human serum albumin (13). Our results are also in close agreement with those obtained on casein, lactoglobulin, and egg albumin by Hac, Snell, and Williams (10) with the microbiological method, and are in fair agreement with the microbiological analysis of casein by Dunn and his colleagues (9). Since our method does not differentiate

TABLE III  
Glutamic Acid Content of Proteins

B. A. PRESCOTT AND

TABLE III

Glutamic Acid Content of Proteins

Protein	Amount hydrolyzed*	Factor	Per cent glutamic acid found								Mean ( $\pm$ standard error)	Final value corrected for cystine†	Values from literature‡			
			per cent													
			21.6	20.3	21.6	22.2	21.0	21.9	20.5	21.3						
Casein	7.81	1.40	21.6	20.3	21.6	22.2	21.0	21.9	20.5	21.3	21.6 $\pm$ 0.2	21.6	22.5 (9) M., 21.7 (10) M.			
	7.51	1.40	21.6	20.3	21.6	22.2	21.0	21.9	20.5	21.3	21.6 $\pm$ 0.2	21.6	15.3 (11)§ Ia., 15.0 (10) M.			
	8.93	1.40	21.6	20.3	21.6	22.2	21.0	21.9	20.5	21.3	21.6 $\pm$ 0.2	21.6	18.1, 18.9 (11)§ Ia., 19.1 (12)			
	15.32	1.40	21.6	20.3	21.6	22.2	21.0	21.9	20.5	21.3	21.6 $\pm$ 0.2	21.6	18.1, 18.9 (11)§ Ia., 19.1 (12)			
Egg albumin	8.80	1.36	21.1	20.3	20.3	20.3	19.9	19.6	19.6	19.6	19.8 $\pm$ 0.2	19.4	I. d., 10.0 (10) M.			
	15.05	1.36	21.1	20.3	20.3	20.3	19.9	19.6	19.6	19.6	19.8 $\pm$ 0.2	19.4	I. d., 10.0 (10) M.			
$\beta$ -Lactoglobulin	15.25	1.36	17.5	17.0	17.7	17.2	17.7	17.3	17.4	17.4	17.0 $\pm$ 0.15	17.1	17.0 (13) I. d.			
	13.08	1.36	18.0	18.1	18.7	18.8	18.3	18.3	17.9	17.9	17.0 $\pm$ 0.15	17.0	17.1 (13) I.			
Bovine serum albumin	14.58	1.40	17.0	17.4	17.4	17.4	17.9	17.9	17.9	17.9	17.7 $\pm$ 0.10	17.0	17.1 (13) I.			
	12.02	1.36	18.3	17.8	18.7	17.9	17.9	17.9	17.9	17.9	17.7 $\pm$ 0.10	17.0	17.1 (13) I.			
Human serum albumin																

\* Amount of protein hydrolyzed in 10 ml. of 6N HCl.  
 † Final value corrected for cystine by using the following factors: Casein, 1.40; Egg albumin, 1.36;  $\beta$ -Lactoglobulin, 1.36; Bovine serum albumin, 1.40; Human serum albumin, 1.36.  
 ‡ Values from literature: (9) M. = M.

\* Corrected for moisture and ash.

† Per cent glutamic acid minus (per cent cystine + cysteine)  $\times$  0.12.

‡ Per cent glutamic acid minus (per cent cystine + cysteine)  $\times$  0.12.

§ M., microbiological method; Ia., isolation; I. d., isotopic dilution and mother liquors.

¶ Glutamic acid isolated without amount estimated in residues and mother liquors.

between *l*- and *d*-glutamic acid, while the isotope dilution method does, the findings indicate that little racemization occurs during hydrolysis.

#### DISCUSSION

Of the two micromethods available for the determination of glutamic acid, Cohen's procedure (14), based on the conversion of glutamic acid into succinic acid, which is estimated enzymatically, is most accurate for amounts above 1 mg. The microbiological method, as carried out at present, covers a slightly higher range of glutamic acid quantities than our procedure. The chemical method described in this paper can be used to determine the amino acid without differentiation between its isomers, and may, therefore, serve as a useful supplement to the microbiological procedure (10).

The specificity of the chemical method rests on different grounds from that of the microbiological procedure. Only substances which are adsorbed and eluted under the same conditions, as is glutamic acid, and which give an acidic hydrazone directly or after reaction with ninhydrin, can interfere. Glutamine is removed to the extent of 99 per cent in our method and therefore it is possible to determine glutamic acid in the presence of a considerable excess of glutamine. In the above procedures glutamine is determined as glutamic acid.

#### SUMMARY

A method for the determination of glutamic acid in the range of 5 to 50  $\gamma$  is described. The acid aldehyde formed from the amino acid by the action of ninhydrin is converted, after removal of unchanged ninhydrin by means of guanidine and lead acetate, into the 2,4-dinitrophenylhydrazone, which is transferred into capryl alcohol and then extracted with borate buffer. The color developed after the addition of sodium hydroxide to the borate solution is measured in a colorimeter.

The synthetic 2,4-dinitrophenylhydrazone of  $\beta$ -formylpropionic acid is used as the standard. The stability of the hydrazone in acid and alkaline solution decreases rapidly with increasing temperature, acidity, or alkalinity, but under the conditions of the method only a negligible amount is destroyed.

Aspartic acid and cystine (or cysteine) interfere in the determination by formation of hydrazones which appear in the borate solution. Aspartic acid is removed by chromatographic analysis on aluminum oxide on a micro scale and elution of glutamic acid with acetic acid. The interference of cystine or cysteine is eliminated by subtraction of 12 per cent of the amount of these amino acids present. Glutamic acid may be determined in the presence of a large excess of glutamine.



The recovery of glutamic acid from pure solutions without chromatographic analysis is quantitative if account is taken of the distribution of the synthetic hydrazone between the solvents used in the procedure. Under the conditions described 14 per cent of the glutamic acid present is retained in the aluminum oxide column.

The glutamic acid content of several proteins was determined; the results are in good agreement with those secured by other reliable methods.

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# THE IMMUNE PROTEINS OF BOVINE COLOSTRUM AND PLASMA\*

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The important rôle of the colostrum in the transmission of antibodies from mother to offspring, and particularly in the protection of the new-born in ruminants to infectious disease, has been shown by the work of many investigators (1). Howe has demonstrated by salt fractionation studies that the serum of the new-born calf taken prior to suckling is deficient in a globulin fraction, and that this globulin, generally associated with antibodies, appears after the calf has ingested colostrum (2). More recent studies by means of the electrophoretic technique of Tiselius have shown that the serum of the new-born calf contains little or no  $\gamma$ -globulin, but that the slow moving globulin component appears after the feeding of colostrum (3).

Early work on the composition of colostrum demonstrated that the protein content was far higher than that of milk, and that the colostrum was particularly rich in a globulin precipitable at low concentrations of salts such as ammonium sulfate. Crowther and Raistrick (4) separated colostrum into the three fractions which have usually been prepared from milk; namely, casein, lactoglobulin, and lactalbumin. Their studies of the nitrogen distribution by the Van Slyke method showed that these fractions could readily be differentiated from one another.

In the present study, colostrum and the protein fractions derived from it have been studied electrophoretically. It was found that an electrophoretically homogeneous globulin could be readily isolated in high yield by the conventional precipitation with ammonium sulfate. This lactoglobulin possesses all of the immune properties of colostrum and has been studied with a view to its characterization by physical and chemical methods. The immune fractions of bovine plasma have also been isolated in order to compare the properties of immune proteins found in different body fluids. It is convenient to refer to the globulins which are associated with immunity and found in colostrum and plasma as "immune globulins." It is fully realized that the immune properties probably account for only a very small part of the total protein.

\* A preliminary report of this work was presented before the Thirty-seventh annual meeting of the American Society of Biological Chemists at Atlantic City, 1946 (*Federation Proc.*, 5, 154 (1946)).

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## EXPERIMENTAL

Electrophoretic studies were performed at 1° in a Tiselius apparatus equipped with the Longworth scanning device. Unless otherwise specified, the solutions were equilibrated by dialysis for at least 48 hours with a veronal (diethyl barbiturate) buffer at pH 8.4 to 8.6 and at an ionic strength of 0.1.

*Fractionation of Colostrum*

In order to study the properties and quantities of the various proteins, the colostrum was arbitrarily separated into several fractions. 2 liters of colostrum collected 1 hour post partum were centrifuged thoroughly and the orange-colored fat layer was discarded. The colostrum (1700 cc.) was then diluted 4-fold with distilled water and slowly adjusted with 0.5 M HCl to pH 4.5. The casein precipitate (Fraction A) was removed by filtration on coarse fluted paper and the solution was clarified by filtration through a thick layer of paper pulp. The filtrate was brought to pH 6.0 with 0.5 M NaOH and successive fractions were removed at 0.3 (Fraction B), 0.5 (Fraction C), and 0.9 (Fraction D) saturation with ammonium sulfate. Fractions B, C, and D were redissolved in water at about 5 per cent concentration and reprecipitated within the same limits of salt concentration after any turbidity present at the lower salt concentration was first removed by filtration. Each of these fractions was dialyzed at 2° until salt-free and then dried by lyophilizing. Fraction A was redissolved with the minimum quantity of 0.1 M NaOH, filtered clear, and then reprecipitated at pH 4.5. This precipitate was washed twice with distilled water, redissolved with the aid of dilute alkali, and then lyophilized.

Fig. 1 shows the electrophoretic patterns obtained with each of the four fractions and with the original colostrum. Table I presents the data for the yield of each fraction and its composition as determined from the electrophoretic pattern. The components included in the vertical columns of Table I are not necessarily to be regarded as the same in all cases but are listed in that manner for tabular convenience. The separate fractions have been designated by the name of the principal protein which was found to be present in each fraction. Fractions A (casein) and D ( $\beta$ -lactoglobulin) will be discussed only briefly and are therefore presented first.

*Casein (Fraction A)*—It is apparent that the crude casein (Fraction A) is complex in nature, like that of milk, and contains at least two components (5). The nature of the third component has not been investigated further but it may be a globulin which precipitated because of the alteration in salt concentration on dilution of the colostrum and adjustment of the pH. The boundary migrating at  $-4.2 \times 10^{-5}$  sq. cm. per volt per second in the whole colostrum is obviously composite in nature and includes casein as well as

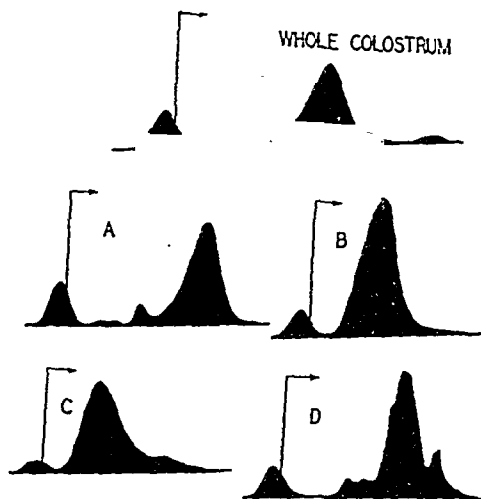


FIG. 1. Electrophoretic patterns of the descending boundaries of whole colostrum and of fractions derived from it. A is the casein; B, C, and D are ammonium sulfate fractions obtained between 0 and 0.3 saturation (B), between 0.3 and 0.5 (C), and between 0.5 and 0.9 (D) respectively. Electrophoresis was for 200 minutes in veronal buffer of pH 8.3 to 8.4 at an ionic strength of 0.1.

TABLE I

*Electrophoretic Composition of Fractions from Colostrum*

These analyses were made from photographs taken after 200 minutes in the Tiselius apparatus at 1° in veronal buffer of pH 8.3 to 8.4 at an ionic strength of 0.1. Mobilities are  $\times 10^{-5}$  sq. cm. per volt per second. The protein content of the whole colostrum was calculated for 1700 cc. of fat-free fluid which contained 3.25 mg. of protein N per cc. (protein factor = 6.4).

Fraction	Dry weight isolated	Immune lactoglobulin												
		gm.	u	per cent	u	per cent	u	per cent	u	per cent	u	per cent	u	per cent
Whole colostrum.....	355				-1.8	54	-2.6	7	-4.2	35	-5.5	2	-7.2	2
A, casein.....	60	-1.3	1		-1.8	1	-2.7	6	-4.0	18	-5.1	74		
B, immune lactoglobulin.....	101				-2.1	100								
C, immune lactoglobulin.....	113				-2.1	85	-3.6	5	-4.6	10				
D, $\beta$ -lactoglobulin.....	17				-2.2	4	-3.2	5	-4.3	75	-5.8	10	-6.3	4
													Higher	2

other components. It should be noted that electrophoresis of whole colostrum or milk has always shown differences in mobility for casein and  $\beta$ -lactoglobulin compared with the mobility for these same components in a more homogeneous state.

*$\beta$ -Lactoglobulin (Fraction D)*—This is a major component of the fraction which is called "lactalbumin" in the older literature. As is shown in Table I, only a small part of the protein in colostrum was isolated by precipitation at high concentrations of ammonium sulfate; this is in agreement with the data of Crowther and Raistrick (4) and Engel and Schlag (6). We have found that 75 per cent of the protein in this fraction, as estimated from the electrophoretic pattern, is the  $\beta$ -lactoglobulin originally isolated from milk by Palmer (7). When Fraction D was thoroughly dialyzed and adjusted to pH 5.2, the globulin crystallized in high yield. It possessed the crystalline form and other properties described by Palmer and others for this protein. A recrystallized sample was homogeneous on electrophoresis in veronal buffer at pH 8.4 and migrated with a mobility of  $-4.9 \times 10^{-5}$  sq. cm. per volt per second. Other preparations of crystalline  $\beta$ -lactoglobulin from milk migrated with mobilities of  $-4.9$  to  $-5.2 \times 10^{-5}$  sq. cm. per volt per second at pH 8.4 to 8.5.

*Immune Lactoglobulin (Fractions B and C)*—The animals from which this and other samples of colostrum were obtained had been hyperimmunized. While the immunity of these animals is the object of a separate study by Dr. August Holm of these laboratories and will be described by him elsewhere, it may be stated that the total immune activity of colostrum is definitely associated with the protein of low electrophoretic mobility ( $-1.8$  to  $-2.2 \times 10^{-5}$  sq. cm. per volt per second at pH 8.4). Immune activity has not been found in fractions free from this protein, and conversely the isolated protein accounts completely for the immune properties of colostrum. It is convenient to refer to this protein from colostrum or milk as immune lactoglobulin; this will serve to distinguish it from  $\beta$ -lactoglobulin.

The immune protein is definitely globulin in character. This is partly indicated by its precipitation at low concentrations of ammonium sulfate, but is more clearly demonstrated by its low solubility in the neighborhood of the isoelectric point, about pH 5.8 to 6.2, and by the marked increase in solubility in the presence of neutral salts. Moreover, as will be discussed below, prolonged dialysis at the isoelectric point causes a precipitation of a water-insoluble portion of the immune lactoglobulin.

It is apparent from Table I that the immune lactoglobulin is the main protein constituent of colostrum as determined both from the electrophoretic analysis of the whole colostrum and from the composition of Fractions B and C. Adding the total yield of Fraction B (101 gm.) and 85 per cent of the yield of Fraction C (96 gm.) shows that 55 per cent of

the original protein in the whole colostrum was isolated as the immune lactoglobulin, as compared with the 54 per cent found by the electrophoretic analysis of the whole colostrum. This surprisingly good recovery shows the great preponderance of this protein compared with any other present in colostrum.

With other samples of colostrum obtained from different animals, it was found that, after removal of the casein as described above, the immune lactoglobulin could be obtained quantitatively from the colostrum whey free of other proteins determined electrophoretically. This was accomplished by precipitation at 0.4 saturation with ammonium sulfate at pH 6.0, solution in water followed by filtration, and reprecipitation at 0.4 saturation. This operation was carried out three times in all; the final precipitate was filtered, pressed free of excess sulfate between thick layers of filter paper, dialyzed thoroughly at 2°, and then lyophilized. From 2 liters of colostrum obtained 1 hour post partum, there were obtained 1295 cc. of fat-free colostrum which contained 40.0 mg. of protein N per cc. or 332 gm. of protein, with the factor 6.4. Since 184 gm. of homogeneous immune lactoglobulin were isolated, the yield was 55 per cent of the total. From this same cow, another 2 liters of colostrum obtained 10 hours post partum gave 1620 cc. of fat-free fluid and from this there were obtained 230 gm. of globulin or 55 per cent of the total protein as before. Although this second milking contained considerably less fat than the first sample, the total protein content and the composition of the aqueous phase remained the same. However, the colostrum of two other cows gave considerably smaller quantities of globulin for the second milking, the yields for which were 12 and 30 per cent of those obtained for the first milking. By the 2nd day, the composition of the colostrum begins to approach that of milk and the immune lactoglobulin fraction can no longer be obtained free of other proteins by the simple method described above. The change in composition from colostrum to milk has been described in detail by Crowther and Raistrick (4) and Engel and Schlag (6).

Exhaustive dialysis of the immune lactoglobulin fraction may result in the separation of water-insoluble and water-soluble or euglobulin and pseudoglobulin fractions. In only one instance was this done; for the other specimens the total immune globulin was used for characterization of these proteins. The euglobulin was separated by centrifuging; it was washed twice in the centrifuge with distilled water at 2° to remove traces of pseudoglobulin and then suspended in water and lyophilized. The pseudoglobulin was diluted 4-fold with distilled water at 2° and then filtered through a sterilizing pad and lyophilized. The ratio of pseudoglobulin to euglobulin was about 3:4. In Fig. 2 the electrophoretic patterns for the euglobulin and pseudoglobulin are shown. These runs

were carried out in veronal buffer at pH 8.5. The pseudoglobulin migrated at a slightly higher mobility than the euglobulin,  $-2.2$  compared with  $-1.9 \times 10^{-5}$  sq. cm. per volt per second. Immune activity was present in both the pseudoglobulin and euglobulin fractions.

The above isolations have clearly demonstrated that the initial colostrum is extraordinarily rich in protein and that the principal protein is the immune lactoglobulin. For the three animals which we have studied, the aqueous phases contained 25.6, 20.9, and 15.4 per cent protein respectively and the immune lactoglobulin accounted for 55, 55, and 41 per cent of the total protein.

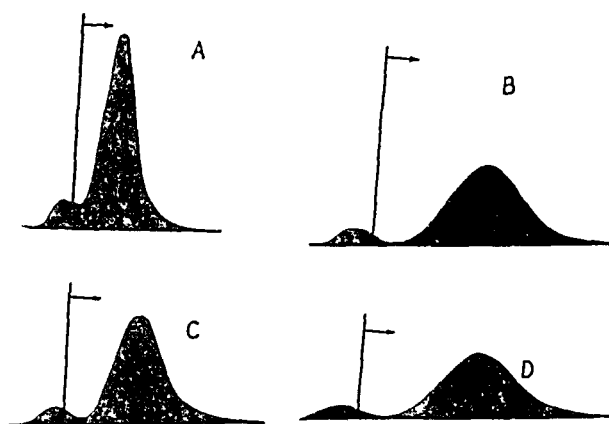


FIG. 2. Electrophoretic patterns of immune globulin from colostrum. *A* and *B*, euglobulin after 100 and 200 minutes; *C* and *D*, pseudoglobulin after 100 and 200 minutes. The runs were performed in veronal buffer of pH 8.5 at an ionic strength of 0.1.

### *Isolation of Plasma Globulins*

It has long been recognized that the colostrum or milk globulin is related to a serum globulin. The immunological studies of Wells and Osborne (8) had clearly shown this, and Crowther and Raistrick had pointed out earlier that lactoglobulin and serum globulin were chemically indistinguishable by the methods available to them. It must be recognized that the proteins studied by these investigators were not homogeneous by present day standards. While there is no doubt of the relationship of the globulins of blood and milk, one cannot assume identity without further study. The plasma of hyperimmunized cows was therefore fractionated to obtain the components with which the immune activity is associated. It was soon recognized that, as in the plasma of the horse (9), the immune activity of bovine plasma is present in two components,  $\gamma$  and T. Both of these



components could be readily isolated by the methods elaborated by Cohn and his collaborators (10, 11) for the fractionation of plasma.

Fraction I (fibrinogen) was precipitated at 8 per cent ethanol and pH 7.3 at  $-2^{\circ}$  from the plasma. The Fraction II + III, which contained most of the T and  $\gamma$  components, was then removed at 25 per cent ethanol and pH 6.9 at  $-5^{\circ}$ . From 24 liters of plasma there were obtained 2900 gm. of wet Fraction II + III paste. This paste was suspended in water and then fractionated by Method 3C (11). The yields of lyophilized powders were 115 gm. of Fraction III-1 and 100 gm. of Fraction II.

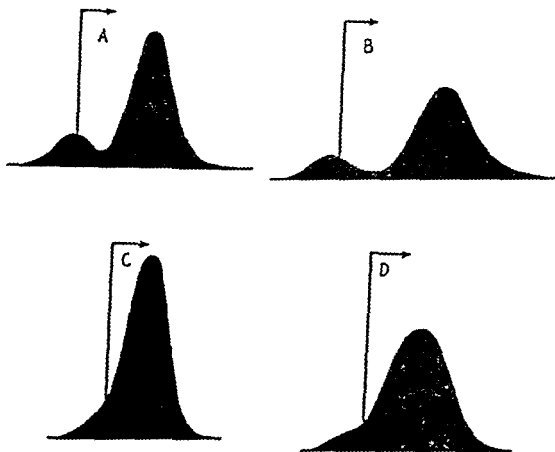


FIG 3 Descending boundaries of electrophoretic patterns obtained with T-globulin (A and B) and  $\gamma$ -globulin (C and D), A and C were photographed after 100 minutes, B and D after 200 minutes. The runs were performed in veronal buffer at pH 8.5 to 8.6 and at an ionic strength of 0.1

On electrophoretic analysis, it was found that reprecipitated Fraction II consisted entirely of  $\gamma$ -globulin of mobility  $-1.1 \times 10^{-5}$  sq cm. per volt per second in veronal buffer of pH 8.5. Fraction III-1 contained 90 per cent T-globulin ( $-2.1 \times 10^{-5}$  sq cm. per volt per second), 9 per cent  $\alpha$ -globulin ( $-4.2 \times 10^{-5}$ ), and 1 per cent albumin ( $-6.5 \times 10^{-5}$ ). A homogeneous T component was obtained by the following procedure. The dried powder was dissolved in water at  $1^{\circ}$  at 2 per cent concentration, adjusted to pH 5.2, and filtered clear. The fraction insoluble in 15 per cent ethanol at  $-2^{\circ}$  was discarded. The T fraction was then precipitated at 25 per cent ethanol and  $-5^{\circ}$ . This precipitate was dissolved in water at  $1^{\circ}$  (2 per cent solution), and the insoluble protein removed by filtration. The T component was precipitated at pH 6.5 and 15 per cent ethanol at

$-2^{\circ}$ , and was lyophilized. It proved to be homogeneous on electrophoresis at pH 8.6, and had a mobility of  $-2.4 \times 10^{-5}$  sq. cm. per volt per second. Fig. 3 shows the electrophoretic patterns obtained with the homogeneous  $\gamma$  and T fractions. Other properties of these proteins will be described below in comparison with the colostrum globulin.

### *Isoelectric Points*

The purified globulins were studied electrophoretically in the Tiselius apparatus with univalent buffers at an ionic strength of 0.1 and at  $1^{\circ}$ .

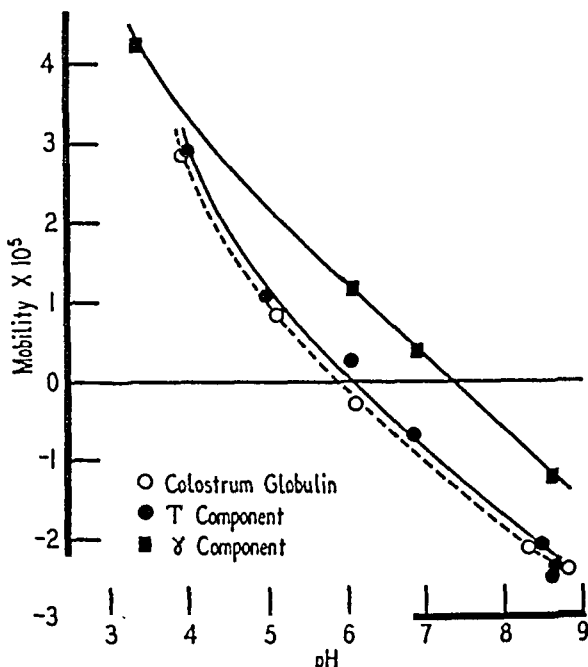


FIG. 4. Electrophoretic mobility as a function of pH for colostrum globulin, T-globulin, and  $\gamma$ -globulin. All of the measurements were calculated from descending migrations in univalent buffers at  $1^{\circ}$ . The mobility is in sq. cm. per volt per second.

At all pH values studied these proteins migrated as single components, although the patterns showed greater symmetrical spreading than would normally be expected for homogeneous proteins. The electrophoretic mobilities calculated from the data are shown in Fig. 4 as a function of the pH. The apparent isoelectric point of the  $\gamma$ -globulin is at pH 7.2, that of the T component at pH 6.15, and of the total immune globulin of colostrum at pH 5.85. For the protein from the colostrum of another animal, the euglobulin was found to have a higher isoelectric point, about pH 6.2, than the pseudoglobulin, which was at pH 6.0. It is apparent from these data

that the T-globulin and colostrum globulin are similar although not necessarily identical in their variation of mobility with pH, while the  $\gamma$ -globulin is distinctly different from either of these. Since the homogeneity of the plasma proteins with respect to sedimentation and solubility is still unknown and the colostrum immune globulin is known to be a mixture, rigid comparisons are not possible at present. The T component does, however, resemble the pseudoglobulin more than it does the euglobulin or total colostrum globulin.

### *Elementary Analyses*

These were performed on the preparations described above. The data given in Table II are for the ash and moisture-free proteins.  $\gamma$ -Globulin A was obtained from slaughter-house blood of steers, while B was from hyperimmune cow blood. The analyses were performed by Mr. J. F. Alicino of The Squibb Institute for Medical Research. No phosphorus

TABLE II

#### *Elementary Analyses of Immune Globulins*

The data are calculated in per cent for the ash- and moisture-free proteins.

Protein	C	H	N (Dumas)	S	Ash
Colostrum pseudoglobulin . .	47.68	7.18	15.44	1.08	0.05
“ euglobulin.... . .	48.50	7.27	15.53	1.09	0.14
Milk pseudoglobulin . . . .	48.09	7.17	15.63	1.09	0.89
Total colostrum globulin. . . .			15.63	1.01	0.18
$\gamma$ -Globulin A..... . .	48.08	7.11	15.75	1.05	0.01
“ B.... . .			15.67	0.99	0.18
T-Globulin..... . .	51.93	7.15	16.01	0.95	0.31

was detected in these proteins colorimetrically after digestion with nitric acid and perhydrol.

### *Diffusion Constants*

These were measured in the electrophoresis cell by the method described by Longworth (12) from photographs taken by the schlieren scanning method. Results were computed by the formula  $D = (A^2)/(4\pi tH^2)$  where  $A$  is the area under the curve,  $H$  the maximum height,  $t$  the time in seconds, and  $D$  the diffusion constant in sq. cm. per second.

The runs were performed in buffers of 0.1 ionic strength at 1° and corrected to water at 20°. Each run was performed in duplicate, both halves of the cell being used separately. From four to six photographs were taken at intervals from about 12 to 60 hours and the values averaged. The data are given in Table III. For comparison with the colostrum globulins, there

are included measurements on the T- and  $\gamma$ -globulins of the cow and the  $\gamma$ -globulin of the horse. The horse globulin was derived from antitetanus serum and was electrophoretically homogeneous.

It is apparent that the diffusion constants found for the colostrum globulins are of the same magnitude as those for the T- and  $\gamma$  globulins and are in good agreement with the results of Pedersen (13) for bovine  $\gamma$ -globulin who found  $D_{20w} = 3.74, 3.58, \text{ and } 3.81$ . Our measurements suggest a small difference in  $D_{20w}$  for the euglobulin and pseudoglobulin, which may be significant. Nevertheless, all three bovine immune proteins are of approximately the same size, and, by analogy with similar proteins, indicate molecular weights in the region of 160,000 to 190,000.

For the horse  $\gamma$ -globulin, Neurath, Cooper, and Erickson (14) found for their GI pseudoglobulin  $D_{20w} = 4.1 \times 10^{-7}$  when their result is corrected for the viscosity difference in water between  $25^\circ$  and  $20^\circ$ . Cohn *et al.*

TABLE III  
*Diffusion Constants of Immune Globulins*

Protein	Concentration	Buffer	pH	$D_{20w} \times 10^7$
	<i>per cent</i>			<i>sq. cm. per sec.</i>
Colostrum A.....	0.4	Veronal	8.45	$3.50 \pm 0.06$
“ “.....	1.2	Cacodylate	6.62	$3.69 \pm 0.11$
“ euglobulin B.....	1.2	Veronal	8.45	$3.34 \pm 0.00$
“ pseudoglobulin B.....	0.8	“	8.46	$3.86 \pm 0.19$
Bovine $\gamma$ -globulin.....	1.8	“	8.37	$3.53 \pm 0.09$
“ T-globulin.....	1.5	“	8.61	$3.60 \pm 0.23$
Horse $\gamma$ -globulin.....	0.6	“	8.43	$4.08 \pm 0.23$
“ “.....	1.6	“	8.54	$3.78 \pm 0.04$

(15) also cite for horse  $\gamma$ -globulin,  $D_{20w} = 4.1 \times 10^{-7}$ . Pappenheimer, Lundgren, and Williams (16) obtained  $4.4 \times 10^{-7}$  for a purified diphtheria antitoxic globulin.

#### *Anaphylactic Tests in Guinea Pigs*

Wells and Osborne (8) carefully reviewed the older data on the immunological relations of the milk and plasma proteins and extended this work, using the milk protein preparations prepared by Osborne and his collaborators. They clearly differentiated by anaphylaxis in guinea pigs the lactoglobulin from casein, lactalbumin, and the alcohol-soluble protein of milk, and showed that only the globulin sensitizes to beef serum or causes reactions in animals sensitized to beef serum. These observations have been confirmed with the homogeneous  $\gamma$ -globulin and colostrum globulin.

Guinea pigs weighing  $300 \pm 10$  gm. were sensitized by the intraperitoneal

injection of 10.8 mg. of colostrum immune globulin in a volume of 1 cc. These animals were tested 30 days later by intravenous injection of  $\gamma$ -globulin and colostrum globulin. For both of these proteins, severe reactions with convulsions were produced at levels of 0.2 to 0.4 mg., and fatal reactions invariably resulted from the injection of 0.5 to 0.6 mg. At the time of injection, the guinea pigs weighed  $390 \pm 20$  gm. No differences in time or quality of response could be observed and the two proteins were quantitatively equivalent.

#### DISCUSSION

It has been amply demonstrated in the past that the principal proteins of milk, casein, and  $\beta$ -lactoglobulin (or lactalbumin), are present only in the mammary secretion, and that these proteins are distinct from any known plasma proteins. However, the presence of immune properties in milk and colostrum raises many questions regarding the relationship of the proteins possessing this function in these secretions compared with the proteins circulating in the blood stream. Although this question has been partially answered by the anaphylactic studies of many investigators, particularly Osborne and Wells, the problem remained unsolved.

In the cow, as in the horse, the immune activity of the plasma is present in at least two well defined components which differ in their electrophoretic mobility and isoelectric points. Although the immune globulin which has now been isolated from colostrum is similar to the T component of plasma in these properties, the two proteins are not identical, as is indicated by differences in amino acid composition and ultraviolet absorption spectra (17, 18). Undoubtedly, all of the proteins concerned with immunity in the same species are closely related (as is partly shown by the anaphylactic studies) but differ somewhat from each other. However, the molecules may vary in composition in portions of the molecule not concerned with either the immune properties or species specificity. That part of the horse antitoxins is not concerned with immune activity is suggested by the studies which have shown that approximately half of the molecule may be digested without impairment of the antitoxic functions of the remaining smaller molecules (19).

The absorption of immune globulin from colostrum into the blood stream of the new-born calf has been shown to produce a new electrophoretic component of plasma (3), which has been called  $\gamma$ -globulin. The studies reported here have demonstrated that the colostrum globulin is readily differentiated electrophoretically from the  $\gamma$ -globulin. It has been found that the absorbed globulin in the serum of the new-born calf possesses the mobility of the colostrum globulin and not that of  $\gamma$ -globulin.<sup>1</sup>

<sup>1</sup> Smith, E. L., and Holm, A., to be published.

From the work reported in this paper on colostrum, it is clear that the older concept of a lactalbumin fraction is not justified. Palmer showed that more than half of the protein in the lactalbumin fraction of milk whey could be obtained as a crystalline globulin. Unpublished data on the electrophoresis of milk whey have shown that about 60 per cent of the total protein is Palmer's  $\beta$ -lactoglobulin. It is not implied that proteins possessing the properties of albumins are not present in milk or colostrum, but it must be emphasized that what has hitherto been called "lactalbumin" is not primarily an albumin at all. The name has been used both for the total coagulable protein of milk whey, which is a complex mixture containing predominantly two or more globulins in addition to a large number of other proteins, or it has been used in referring to a "lactalbumin fraction"

TABLE IV  
*Some Properties of Two Globulins of Milk and Colostrum*

	$\beta$ -Lactoglobulin	Immune lactoglobulin
Isoelectric point, <i>pH</i> .....	5.2	5.85
Mol. wt.....	42,000	Ca. 160,000-190,000
$D_{20w}$ .....	$7.3 \times 10^{-7}$	$3.6 \times 10^{-7}$
Carbohydrate.....	None	Present
Sulfur, %.....	1.60	1.07
Leucine, %.....	15.6	8.9
Valine, %.....	5.83	10.2
Tryptophane, %.....	1.94	2.74
Phenylalanine, %.....	3.54	3.6
Dialysis at isoelectric point.....	Crystals	Insoluble euglobulin and soluble pseudo- globulin
Precipitation with $(\text{NH}_4)_2\text{SO}_4$ ....	Ca. 0.4-0.7 saturated	Ca. 0.25-0.4 saturated
Immune activity.....	None	Present

which contains a wide variety of proteins in small amount in addition to  $\beta$ -lactoglobulin. It is only necessary to recall some of the proteins isolated in recent years from bovine whey, *i.e.* a flavoprotein (xanthine oxidase), a copper-protein, a peroxidase, as well as others.

In order to show clearly the distinctive properties of the globulin whose isolation has been described above, some of the properties of  $\beta$ -lactoglobulin and the immune protein are summarized in Table IV. These substances are readily differentiated by their size, carbohydrate content, solubility, and analytical composition with respect to sulfur content and the few amino acids for which data have been obtained on the total immune protein of colostrum (17). The analytical data for  $\beta$ -lactoglobulin have been taken from the paper by Brand and his collaborators (20).

It is a pleasure to acknowledge the indebtedness of the author to Dr. August Holm for making available the colostrum and bovine plasma used in this study and for his cooperation. Gratitude is also due to Leo Zuckerman and Douglas M. Brown for their technical assistance, and to T. D. Gerlough for his help and cooperation.

#### SUMMARY

1. Electrophoretic analysis and isolation have shown that the immune lactoglobulin is the predominant protein in bovine colostrum. This protein has been isolated in electrophoretically homogeneous form.

2. The immune activity of bovine plasma is present in both T and  $\gamma$  components. Both of these have been isolated and characterized in comparison with the colostrum globulin by their elementary composition, isoelectric points, and diffusion constants.

3. The colostrum immune globulin and the plasma  $\gamma$ -globulin have been shown to be quantitatively equivalent in producing anaphylaxis in guinea pigs.

4. The relationship of the various immune proteins has been discussed and it has been pointed out that, while the colostrum globulin is closely related to the  $\gamma$ - and T-globulins, they are not identical. The immune lactoglobulin of colostrum has also been shown to be easily distinguished from  $\beta$ -lactoglobulin.

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# AMINO ACID AND CARBOHYDRATE ANALYSES OF SOME IMMUNE PROTEINS\*

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In recent years it has become evident that antibodies are protein in nature and characteristically associated with certain fractions of plasma. This advance in knowledge has been achieved mainly by the application of physicochemical methods such as studies in the ultracentrifuge and electrophoresis apparatus, and to a more limited extent by analysis of such proteins. This has been due in large part to the small quantities of specifically precipitated antibodies which have been available, and to the difficulty in obtaining these antibodies free from the antigen used for the precipitation, since in most cases the antigen is itself a protein or is highly complex in nature. However, a different approach is possible if the recently expressed view is correct that antibodies are modified globulins which differ from normal globulins only in some specific arrangement or pattern (1). If this is so, analyses of homogeneous globulin fractions of the same electrophoretic mobility should yield identical amino acid analyses regardless of the amount or kind of antibody specificity in the protein analyzed. However, even if this assumption is proved to be invalid, so few analyses are available for immune fractions that knowledge of the composition of these globulins will be valuable for the characterization of the plasma components of different species, and for many other purposes. As a result of isolation studies of proteins concerned with immunity, we have had available sufficient quantities of electrophoretically homogeneous proteins for some analytical studies. This paper will deal with plasma proteins from the horse, cow, and human, and with the immune lactoglobulin of bovine colostrum and milk.

## EXPERIMENTAL

All of the proteins used for the present study were prepared in this laboratory. The bovine proteins were those described earlier (2). The  $\gamma$ -globulin preparation A was derived from steer blood obtained at the slaughter-house;  $\gamma$ -globulin B was processed from the plasma of cows hyper-

\* A preliminary report on a part of this work was presented before the Thirty-seventh annual meeting of the American Society of Biological Chemists at Atlantic City, 1946 (Smith, E. L., *Federation Proc.*, 5, 154 (1946)).

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immunized with mixed antigens. The two  $\gamma$ -globulin preparations have not shown significant differences in composition and will be discussed together. The colostrum and T-globulins were from the same hyperimmune animals, and the milk pseudoglobulin was obtained from normal milk.

The human  $\gamma$ -globulins were electrophoretically homogeneous proteins prepared by Method 9 of Oncley and his collaborators (3) from normal human blood. Aside from the differences in solubility and in method of isolation of these proteins the II-1,2 globulin had an electrophoretic mobility of  $-1.2 \times 10^{-5}$  sq. cm. per volt per second and the II-3 globulin  $-1.5 \times 10^{-5}$  as measured in veronal buffer of 0.1 ionic strength at pH 8.5.

The horse  $\gamma$ -globulin was isolated by alcohol precipitation Method 9 (3), and represents a II-3 fraction. It was found to be electrophoretically homogeneous over a wide pH range with a mobility of  $-1.3$  at pH 8.4 in veronal buffer of 0.1 ionic strength. The T component was isolated by

TABLE I

*Elementary Analysis of Immune Proteins*

The data are given in per cent for the anhydrous ash-free protein.

Protein	C	H	N (Dumas)	S
Horse $\gamma$ -globulin.....	48.15	7.18	15.97	1.00
“ T-globulin.....	47.78	6.38	15.35	0.69
Human $\gamma$ -globulin II-1,2.....	49.78	7.25	15.65	1.00
“ “ II-3.....	49.92	6.99	15.71	0.99

reprecipitation from a III-1 fraction by Method 3C (3). On electrophoresis at pH 8.4 it had a mobility of  $-1.7$ , but it showed a distinct asymmetry, indicating the presence of a small quantity of a more rapidly migrating component, probably  $\beta$ -globulin. Both the  $\gamma$ - and T-globulins were isolated from plasma containing tetanus antitoxin activity, and these globulins were high in antitoxic protection for guinea pigs. The immune and other properties of these proteins will be reported elsewhere.

*Elementary Composition*—The analyses of the bovine proteins used in this study have already been reported (2). In Table I are presented the data obtained for the human and horse proteins. The values given are for the anhydrous ash-free proteins. These analyses were performed by Mr. J. F. Alicino of The Squibb Institute for Medical Research. Phosphorus was not found in these proteins by the usual colorimetric tests after digestion.

The elementary composition reveals a striking difference in the sulfur content of the two horse globulins and affords the first indication of a dif-

ference in the chemical characteristics as distinguished from their physical and immunological properties. We have found the two human proteins to be very similar in their composition. Brand, Kassell, and Saidel (4) obtained 16.03 per cent for the nitrogen and 1.02 per cent for the sulfur content of a II-1 fraction of human  $\gamma$ -globulin; both these values are 2 per cent higher than ours. Since the proteins analyzed were prepared in somewhat different ways, we cannot be certain whether these differences are experimental or due to variation in the proteins themselves.

**Carbohydrate Content**—All of the immune proteins were found to contain carbohydrate. The hexose was estimated colorimetrically with glucose as

TABLE II  
*Carbohydrate Content of Immune Proteins*

The analytical data given below are expressed on a dry ash-free basis.

Protein	Hexose		Hexosamine		Ratio of hexosamine to hexose
	No. of determinations	Content	No. of determinations	Content	
		<i>per cent</i>		<i>per cent</i>	
Colostrum euglobulin.....	3	$2.93 \pm 0.18$	4	$1.53 \pm 0.03$	0.54
“ pseudoglobulin ...	3	$2.52 \pm 0.13$	3	$1.52 \pm 0.05$	0.60
Milk pseudoglobulin... ..	2	$2.96 \pm 0.16$	4	$1.45 \pm 0.03$	0.49
Colostrum total globulin A....	3	$2.63 \pm 0.08$	3	$1.47 \pm 0.09$	0.55
“ “ “ B	2	$2.62 \pm 0.10$	4	$1.49 \pm 0.06$	0.57
Bovine $\gamma$ -globulin A. ...	4	$2.05 \pm 0.09$	4	$1.26 \pm 0.05$	0.61
“ “ B.. .	5	$2.04 \pm 0.05$	3	$1.35 \pm 0.10$	0.66
Bovine T-globulin...	3	$2.51 \pm 0.17$	3	$1.50 \pm 0.04$	0.60
Horse $\gamma$ -globulin... ..	4	$2.53 \pm 0.17$	7	$1.18 \pm 0.13$	0.47
“ T-globulin... ..	3	$2.53 \pm 0.06$	3	$1.53 \pm 0.02$	0.59
Human $\gamma$ -globulin II-1,2 ....	3	$2.29 \pm 0.10$	4	$1.27 \pm 0.09$	0.55
“ “ II-3	3	$2.34 \pm 0.10$	4	$1.23 \pm 0.03$	0.53

a standard by the orcinol method of Tillmans and Philippi (5). Hexosamine was determined by the Elson and Morgan method under the conditions specified by Palmer, Smyth, and Meyer (6). The photometric densities were measured at  $540\text{ m}\mu$  with a Beckman spectrophotometer, with hexosamine standards at three concentration levels. Standards were run with each set of determinations independently and these were found to vary in absolute extinction coefficient by about 3 per cent with fresh Ehrlich's reagent. The data for the carbohydrate estimations are given in Table II.

Carbohydrate has previously been reported to be present in immune proteins (7). Our results demonstrate that carbohydrate is universally

found in these proteins and that there is roughly twice as much hexose as hexosamine. The uncertainty in the hexose estimations is principally due to the selection of a suitable standard. The colors developed by the various proteins were found to differ somewhat and may indicate that different hexoses or hexose combinations are present. In order to avoid the use of different standards based on the problematical identification of these sugars by difference in color or rate of color development, the quantities were estimated in terms of glucose as measured in a visual colorimeter. Pending the actual isolation of these sugars, we believe it is probably safe to assume that there is twice as much hexose as hexosamine, as has already been observed for crystalline egg albumin (8). From the quantitative viewpoint, the estimations of hexosamine are far more reliable and show definite though minor differences for several of these proteins.

*Tryptophane Estimations*—Two different methods were used for the estimation of this amino acid, the microbiological assay described by Greene and Black (9) using *Lactobacillus arabinosus* 17-5, and the Bates (10) colorimetric method. The microbiological method has been shown to give reliable results in agreement with various colorimetric methods on a variety of proteins. For these assays, the proteins were hydrolyzed with  $\text{Ba}(\text{OH})_2$  and the values obtained were multiplied by 2, assuming complete racemization of the tryptophane during the alkaline hydrolysis. For the microbiological determination of tryptophane as well as for the other amino acids assayed by this procedure, values obtained for each hydrolysate were considered to be a single determination, although several measurements were obtained at different concentration levels.

The Bates method possesses the advantage of permitting simple and rapid estimations to be made on the unhydrolyzed protein. It has recently been used for tryptophane determinations by Sullivan and Hess (11) who showed for several proteins that identical results were obtained by this method as compared with others. Our colorimetric estimations were made exactly as described by Sullivan and Hess (11) with a tryptophane standard prepared in the same way as the protein, and also a protein standard of accurately known tryptophane content. The latter technique possesses an advantage, inasmuch as no heterochromaticity is observed in matching the two samples. However, since no significant difference in results was found with the two standards, all of the measurements have been averaged.

The data for the tryptophane contents of the immune proteins are given in Table III. In general, the two different methods of assay give results in close agreement. One peculiarity is the fact that the euglobulin and pseudoglobulin preparations from colostrum show lower tryptophane content than the preparations from colostrum containing both globulins but

TABLE III

*Tryptophane Content of Immune Proteins*

The values given are for the anhydrous ash-free proteins.

Protein	Colorimetric		Microbiological (2 determinations each)	Average
	No. of determinations	Content		
				per cent
Colostrum euglobulin.....	4	2.42 ± 0.25	2.36 ± 0.21	2.39
"    pseudoglobulin.....	4	2.33 ± 0.05	2.20 ± 0.07	2.27
Milk pseudoglobulin.....	2	2.62 ± 0.20	2.78 ± 0.04	2.70
Colostrum total globulin A.....	5	2.49 ± 0.04	2.76	
"    "    "    B.....	3	2.71 ± 0.17	2.98 ± 0.04	2.74
Bovine γ-globulin A.....	2	2.62 ± 0.05	2.55 ± 0.05	
"    "    B.....	3	2.57 ± 0.11	2.72 ± 0.06	2.62
"    T-globulin.....	3	2.64 ± 0.12	2.56 ± 0.02	2.60
Horse γ-globulin.....	4	2.80 ± 0.04	2.66 ± 0.09	2.73
"    T-globulin.....	3	2.88 ± 0.09	2.78 ± 0.13	2.83
Human γ-globulin II-1,2.....	5	2.61 ± 0.03	2.50 ± 0.03	2.56
"    "    II-3.....	3	2.85 ± 0.04	2.76 ± 0.05	2.81

TABLE IV

*Leucine, Valine, and Phenylalanine Content of Immune Proteins*

The analytical data are given for the anhydrous ash-free proteins.

Protein	Leucine		Valine		Phenylalanine (2 determina- tions each)
	No. of determi- nations	Content	No. of determi- nations	Content	
		per cent		per cent	
Colostrum euglobulin..	4	10.4 ± 0.5	4	10.4 ± 1.0	3.62 ± 0.10
"    pseudoglobulin	4	8.5 ± 0.5	4	9.1 ± 0.3	3.68 ± 0.01
Milk pseudoglobulin..	2	9.6 ± 0.3	2	9.6 ± 0.0	3.88 ± 0.12
Colostrum total globulin A.	4	9.3 ± 0.6	3	10.2 ± 0.2	3.79 ± 0.10
"    "    "    B	2	8.5 ± 0.3	4	10.2 ± 0.5	3.57 ± 0.03
Bovine γ-globulin A..	4	7.5 ± 0.3	4	9.6 ± 1.3	3.06 ± 0.13
"    "    B.....	4	7.2 ± 0.2	4	10.4 ± 0.3	3.31 ± 0.05
"    T-globulin..	2	8.6 ± 0.1	2	9.5 ± 0.5	4.48 ± 0.02
Horse γ-globulin.....	4	9.0 ± 0.3	4	10.1 ± 0.8	4.44 ± 0.09
"    T-globulin.....	4	7.5 ± 0.2	4	10.4 ± 0.6	4.08 ± 0.05
Human γ-globulin II-1,2..	4	9.3 ± 0.4	4	9.7 ± 0.5	4.45 ± 0.12
"    "    II-3.....	4	9.5 ± 0.9	4	9.7 ± 0.4	4.69 ± 0.07

obtained from two other cows. The analysis of milk pseudoglobulin is in agreement with the values for the total colostrum globulin. We are at a loss to explain these results except for possible tryptophane destruction

during prolonged dialysis in the absence of salt, which is unlikely, or a variation in the immune proteins of the different animals. As would normally be expected, the analyses for leucine, valine, and phenylalanine (Table IV) show that the results for the total globulin are intermediate between the values for the euglobulin and pseudoglobulin when these differ. We can only record these abnormal tryptophane values and emphasize that they may not be truly characteristic of these proteins.

*Leucine, Valine, and Phenylalanine Estimations*—These amino acids were assayed by the microbiological procedure described by Schweigert, McIntire, Elvehjem, and Strong (12) using *Lactobacillus arabinosus*. Protein hydrolysates were prepared by adding 40 cc. of 2 M HCl to 200 to 500 mg. of protein and autoclaving the mixture at 120° for 5 hours. After hydrolysis, the solutions were neutralized, made up to a definite volume, and compared with standards. The amino acids for the standards were *dl*-valine (Merck), *dl*-phenylalanine (Merck), and *l*-leucine (Merck); the last was sulfur-free and possessed the correct rotation for *l*(-)-leucine. As with microbiological assays for tryptophane, each determination represents a separate hydrolysate. The results are given in Table IV. In general, the reproducibility of these data has been good, with only a few of the determinations showing deviations greater than 5 per cent.

#### DISCUSSION

What is at first sight most striking about the analytical data for all of these proteins is the great similarity in their composition. As with other series of related proteins from different species, there seem to be a fundamentally similar pattern of composition and small variations within this pattern. As examples from the more recent literature, there may be cited the analyses of some crystalline proteins of cucurbit seed (13) and the serum albumins of human, cow, and horse plasma (4).

The two human proteins, which differ in solubility and electrophoretic mobility, are apparently identical in elementary composition, carbohydrate, leucine, valine, and phenylalanine content. However, there appears to be a constant small difference in their content of tryptophane. Brand, Kassell, and Saidel (4) reported 2.86 per cent tryptophane for a human  $\gamma$ -globulin II-1 preparation; this is in good agreement with our data for the II-3 fraction. Brand (14) also found 9.3 per cent leucine, 9.7 per cent valine, and 4.6 per cent phenylalanine for the same II-1 preparation. These values are in excellent agreement with the results reported in Tables III and IV and serve to establish the basic similarity of the different proteins analyzed as well as to confirm the results already obtained.

It is known that human  $\gamma$ -globulin has a molecular weight in the range between 150,000 and 180,000, and Brand has used two different figures for the calculated molecular weight, 171,000 (4) and 156,000 (14). It is of

some interest to examine the implications of our tryptophane analyses. Although our colorimetric and microbiological estimations for the two human  $\gamma$ -globulins differ by about 4 per cent, the difference between the two preparations by each analytical method is the same; namely, 0.25 per cent. This means that, if the two proteins possess the same molecular weight, they differ in tryptophane content by exactly 1 residue. If the  $\gamma$ -globulins have the same number of tryptophane residues, the molecular weights must differ by 9000 or 10,000, depending on whether 22, 23, or 24 is finally taken as the correct number of residues. It may be remarked that the difference in tryptophane content has also been detected in the ultraviolet absorption spectra of the two proteins (15).

The two horse globulins show marked differences in their analytical composition. Most striking are the differences in total sulfur and in leucine, and a less marked although apparently real difference in phenylalanine. The two proteins are alike in tryptophane and carbohydrate content. It does not appear fruitful at the present time to analyze these differences further, since the T component is not completely homogeneous.

It is noteworthy that the analyses of the bovine  $\gamma$ -globulins from normal and hyperimmune blood do not reveal any significant difference. Bovine  $\gamma$ -globulin differs from the T-globulin in leucine and phenylalanine content. It is also possible to differentiate the T-globulin from the colostrum immune globulin. These proteins, which have been shown to be so similar in electrophoretic mobility, isoelectric point, and other properties, can be distinguished with certainty on the basis of the difference in phenylalanine content, although other less striking differences are apparent. The analytical differences in these proteins are reflected in their ultraviolet absorption spectra (15).

From these data, we may safely conclude that the three proteins known to be associated with immune activity in the cow,  $\gamma$ - and T-globulins of plasma and colostrum globulins, possess somewhat different compositions, although they are closely related. Similarly, the proteins of the horse and human also manifest these differences to greater or lesser degree and may be expected to show even more variations as our knowledge is enlarged, although all may be expected to show the same type of fundamental composition.

The technical assistance of Lois Herbert, Rachel Mato, and Helen Zeveney in performing the microbiological assays is gratefully acknowledged.

#### SUMMARY

Amino acid and carbohydrate analyses have been performed on several immune proteins from the cow, human, and horse. While these proteins

show a fundamentally similar constitution, sufficient variations in composition have been found so that all of them may be distinguished chemically. These proteins contain carbohydrate with an approximate ratio of 2 residues of hexose to 1 of hexosamine. The moderately high tryptophane (about 2.3 to 2.9 per cent) and valine (about 9.1 to 10.4 per cent) contents are noteworthy.

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# THE ABSORPTION SPECTRA OF IMMUNE PROTEINS

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Since the absorption spectra of simple proteins are known to depend on their content of the three aromatic amino acids, tyrosine, tryptophane, and phenylalanine, it was of interest to examine the group of purified immune proteins which were available in order to determine whether differences in their composition could be found by this method. These proteins, all of which are associated with immune properties in the plasma of the cow, horse, and human and the colostrum of the cow, have been described in terms of the method of isolation employed and of their physical and chemical properties (1, 2). It has already been shown that, even in the same species, proteins of quite different composition are associated with immune properties. These differences are also reflected in the absorption spectra of the proteins.

## EXPERIMENTAL

The preparation and properties of the proteins used in this study have already been described (1, 2). For measurement of the absorption spectra, the proteins were dissolved in 0.15 M NaCl, adjusted with 0.1 M NaOH to pH  $7.0 \pm 0.3$ , and then made up to a definite volume. The protein concentrations were calculated on an ash- and moisture-free basis, and checked by nitrogen determinations. The measurements were made at room temperature with a Beckman spectrophotometer having a hydrogen lamp as light source.

The absorption spectra of the proteins studied are shown in Figs. 1 and 2. Since all of these proteins have been shown to contain tyrosine, tryptophane, and phenylalanine,<sup>1</sup> the typical protein absorption band with a broad maximum at about 280 m $\mu$  was to be expected. No evidence was found for the presence of groups that absorb light in the visible or ultraviolet regions of the spectrum other than those of the three aromatic amino acids.

The two human  $\gamma$ -globulin fractions have already been shown to differ slightly in their content of tryptophane, 2.81 per cent for the II-3 and 2.56 for the II-1 fraction. Interestingly enough, this difference is shown in the

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<sup>1</sup> All of these proteins have been analyzed for tryptophane and phenylalanine (2), and tyrosine has been qualitatively demonstrated (unpublished observations)

two absorption spectra (Fig. 1). The two horse serum proteins, which are quite dissimilar in composition and other properties, show this in their absorption curves only in the extinction coefficients and not in the character of the curves.

The most striking differences have been found for the bovine proteins (Fig. 2). The T component is clearly and radically different in its spectrum from either the  $\gamma$ -globulin or the colostrum proteins. Since the absorption band in the region 240 to 250  $m\mu$  is attributed mainly to phenylalanine (3, 4), the steep end-absorption of the T-globulin indicates a higher phenyl-

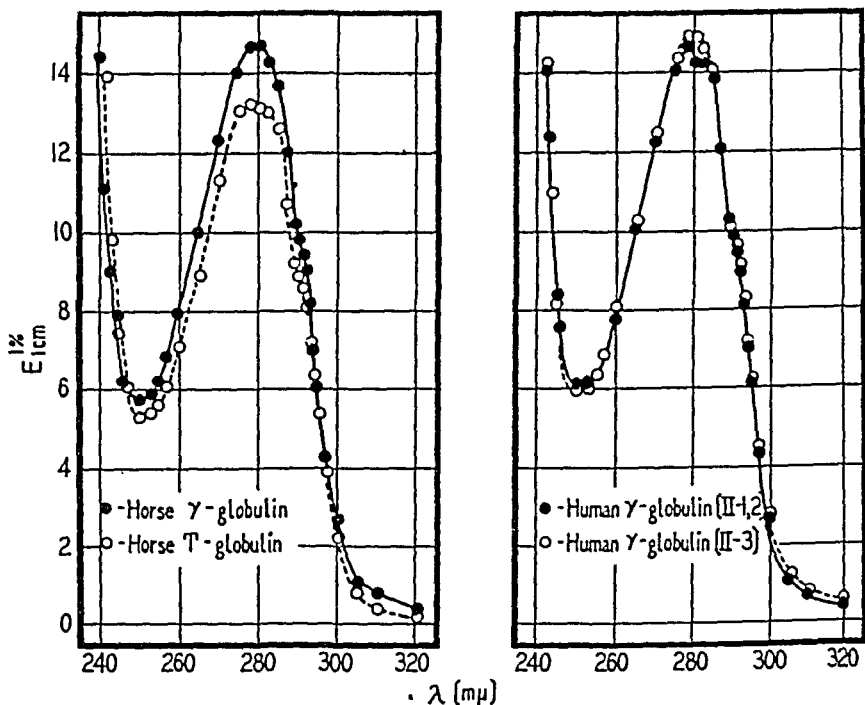


FIG. 1. Ultraviolet absorption spectra of horse and human immune globulins

alanine content than the other proteins. This conclusion is supported by the analytical results, since the preparation contains 4.5 per cent compared with 3.2 per cent for the  $\gamma$ -globulin, and 3.7 per cent for the colostrum globulins. The large differences in extinction coefficients for the total compared with the pseudoglobulin and euglobulin fractions from other animals are to be expected from the earlier finding of differences in tryptophane content.

Coulter, Stone, and Kabat (4) compared the ultraviolet absorption curves of horse pseudo- and euglobulins with pneumococcus Type I antibody prepared by the method of Felton. They found that the antibody re-

sembled the normal pseudoglobulin but showed some characteristic differences. Since the sulfate precipitation method used by these investigators for the preparation of the horse globulins is now known to yield mixtures of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins (5), their results cannot be regarded as evidence of a difference between the antibody and normal globulin. This is likewise true for all of the earlier comparative analyses of normal and immune proteins. Such data are valid only when completely homogeneous preparations of the same globulin fraction from normal and hyperimmune

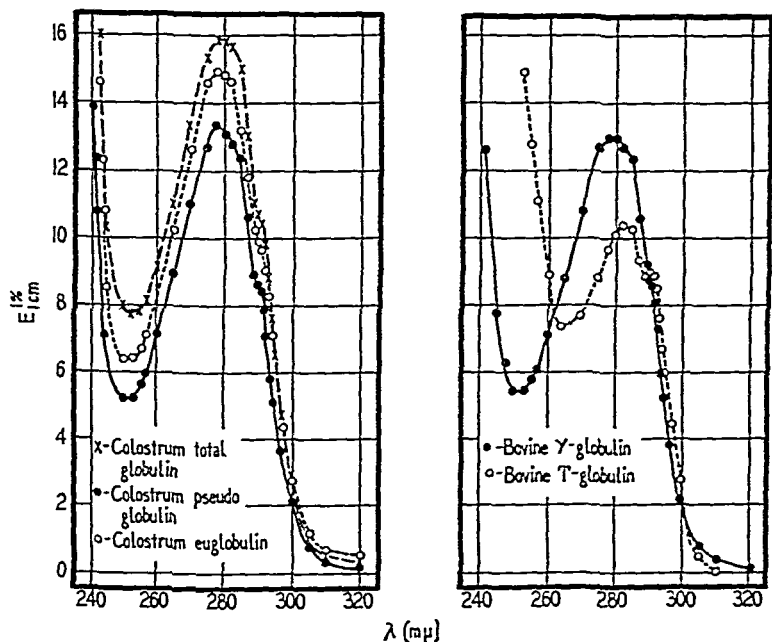


FIG. 2. Ultraviolet absorption spectra of bovine immune globulins

animals are compared. The present results have clearly shown large differences between the different globulin fractions associated with immunity in the same species, and have failed to indicate significant differences in the composition of bovine  $\gamma$ -globulins from normal and hyperimmune plasmas (2).

#### SUMMARY

The ultraviolet absorption spectra of electrophoretically homogeneous proteins associated with immunity from the horse, cow, and human have

been measured. No evidence was found for the presence of light-absorbing groups other than the three aromatic amino acids, phenylalanine, tyrosine, and tryptophane. The differences in spectra measured for these proteins are readily accounted for in terms of their content of these amino acids.

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# EXPERIMENTALLY INDUCED CHANGES IN THE PROTHROMBIN LEVEL OF THE BLOOD

## III. PROTHROMBIN CONCENTRATION OF NEW-BORN PUPS OF A MOTHER GIVEN DICUMAROL BEFORE PARTURITION\*

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Little is known concerning the basic causes that govern the organism's susceptibility to dicumarol.<sup>1</sup> In an attempt to seek new information on this question, the effect of the drug on new-born pups was investigated. Schofield (2) recognized that the new-born were much more highly susceptible to toxic sweet clover hay than were mature animals. He cites the case of a calf (born of a cow fed spoiled sweet clover hay) that developed within a few hours typical symptoms and died while the mother remained well. Roderick and Schalk (3) record a similar instance; the calf was strong and vigorous at birth but the next day was dull and had a puffy swelling above the left hock. Death occurred 28 hours after birth. The mother was continued on sweet clover hay for 7 weeks without signs of hemorrhage.

These studies were carried out before quantitative methods for the determination of prothrombin were developed. It seemed desirable to reinvestigate this problem by using dicumarol instead of toxic sweet clover hay with the particular objective of comparing the prothrombin level of the new-born with that of the maternal organism.

### EXPERIMENTAL

Dogs were selected since new-born pups are sufficiently large to permit taking daily samples of blood. The pregnant dog was fed dicumarol daily during the last week of gestation. Blood from the pups was obtained from the external jugular vein. The prothrombin was determined by the writer's method (4). Analyses were carried out immediately after the blood was collected.

### DISCUSSION

Before studying the effect of dicumarol on the new-born pups, it was necessary to ascertain whether the prothrombin was normally diminished at

\* A preliminary abstract has appeared (*Federation Proc.*, 4, 101 (1945)).

<sup>1</sup> The trade name for 3,3'-methylenebis(4-hydroxycoumarin), the toxic principle which Stahmann, Huebner, and Link (1) isolated from spoiled sweet clover hay.

birth. Link (5) has remarked that the young of dogs and rats show a hypoprothrombinemia during the first few days of life. The results recorded in Table I confirm his statement. The prothrombin concentration of six pups was 40 to 45 per cent of normal several hours after birth and became normal in about 48 hours.

In these determinations, dilution of plasma was found unnecessary. In fact, it was observed that plasma diluted with saline showed an abnormally delayed prothrombin time compared with the sample diluted with plasma treated with aluminum hydroxide. This suggests that dilution by disturbing the physicochemical equilibrium may cause an appreciable error in the determination of prothrombin.

From a practical view this hypoprothrombinemia is insignificant, since these pups still had twice as much prothrombin as is found in normal human

TABLE I  
*Prothrombin Time of New-Born Pups*

Pup No.	Weight	Prothrombin time		
		6 hrs.*	24 hrs.	48 hrs.
	<i>gm.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
1	290	7.5 (45)	7 (50)	6 (100)
2	275	7.5 (45)	7.5 (45)	6.3 (85)
3	297	8 (40)	6.5 (75)	6 (100)
4	305	8 (40)	7.5 (45)	6.5 (75)
5	265	7.5 (45)	7 (50)	6.3 (85)
6	255	7.5 (45)	7 (50)	6 (100)
Mother	7000	6 (100)	6 (100)	6 (100)

The figures in parentheses are the prothrombin concentrations in per cent of normal.

\* After birth.

blood. A hemorrhagic disease of the new-born in pups comparable to the human disease is inconceivable. Physiologically, however, this temporary reduction in prothrombin is of special interest. Since the prothrombin of the mother was normal, it is logical to conclude that the pups *in utero* manufacture their own prothrombin. It seems justifiable to assume that the fetal organism receives relatively as much vitamin K as the maternal. The reduced concentration at birth is therefore probably due to the higher metabolic demands of the fetus for prothrombin. Apparently the amount of vitamin K is inadequate to bring the prothrombin to the normal level until 48 hours or more after birth. Whether this restoration of the prothrombin concentration is due to the vitamin K content of the milk or to the establishment of the bacterial flora in the intestines of the pups is a question that requires further study.

The pups born of a mother fed dicumarol have a prothrombin concentration strikingly lower than that of the mother. From the data of Table II, it will be seen that while the prothrombin time of the maternal blood was 12 seconds (15 per cent of normal) that of the pups varied from 48 to 75 seconds, which indicates a prothrombin concentration of 1 per cent or less. The results of Table III are even more striking. The blood of the pups taken about 1 hour after birth showed a prothrombin time of 100 to 240 seconds, which is less than 0.5 per cent of normal. The maternal prothrombin time was 21 seconds, which is 4 per cent of normal. In 24 hours a further drastic drop in prothrombin occurred in the pups, while in the mother the level remained the same. At this time three of the pups were

TABLE II  
*Prothrombin Time of Pups Born of Mother Fed Dicumarol*

Pup No.	Weight	Prothrombin time							
		2nd day	3rd day*	4th day	5th day	7th day†	8th day	9th day	10th day
	gm.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1	360	48 (1)	27 (4)	32 (3)	22 (5)	7.5 (45)	9 (30)	7.5 (45)	6.5 (75)
2	280	52 (1)	31 (3)	27 (4)	27 (4)	8 (40)	9 (30)	7.5 (45)	6.5 (75)
3	350		36 (2)	39 (2)	25 (4)	9.5 (25)	10 (23)	8 (40)	6 (100)
4	370			33 (3)	26 (4)	8 (40)	8.5 (35)	7.5 (45)	6.5 (75)
5	375		26 (4)	29 (3)	21 (5)	8 (40)	8.5 (35)	7 (50)	6 (100)
6	310	75 (1)		34 (2)	28 (3)	8 (40)	9 (30)	8 (40)	10 (23)
7	315	72 (1)			21 (5)	8.5 (35)	9 (30)	6.5 (75)	6 (100)
Mother‡	8000	12 (15)	12 (15)	18 (7)	22 (5)	8.5 (35)	13 (13)	18 (7)	21 (5)

The figures in parentheses are the prothrombin concentrations in per cent of normal.

\* On the 3rd day Pups 1, 2, and 3 received 2.5 mg. of synkayvite (Hoffmann-La Roche) orally and Pups 6 and 7 were given 25 mg. of ascorbic acid.

† On the 7th day the mother received 120 mg. of dicumarol orally.

‡ 30 mg. of dicumarol were given daily for 4 days before parturition.

given synthetic vitamin K<sup>2</sup> by mouth and intravenously. The following day the prothrombin was slightly elevated in the treated animals, while the untreated pups were dead. Out of a litter of seven four died of hemorrhage. In three the abdomen was filled with blood, while in the fourth there was intramuscular bleeding but not enough to account for the drastic anemia observed. Obviously the fetal and the new-born organisms are more susceptible than the maternal, since the concentration of dicumarol

\* The compounds used were menadione which is 2-methyl-1,4-naphthoquinone, and synkayvite (Hoffmann-La Roche) which is 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt. The latter compound was kindly furnished Hoffmann-La Roche, Inc., Nutley, New Jersey.

which reaches the fetus is certainly not greater than that circulating in the mother's blood.

The effect of synthetic vitamin K in counteracting the hypoprothrombinemia induced by dicumarol is difficult to demonstrate either in pups or adult dogs. While it is true that only the pups that received vitamin K (in the litter recorded in Table III) survived, more extensive studies are needed, since this result may have been accidental. The findings recorded in Table II, as well as unpublished data, fail to demonstrate any marked effect of vitamin K to counteract the action of dicumarol in dogs.

TABLE III

*Severe Hypoprothrombinemia in New-Born Pups from Mother with Only Mild Reduction of Prothrombin Induced by Dicumarol*

Pup No.	Weight	Prothrombin time						
		0 day*	1st day†	2nd day	3rd day	6th day	8th day‡	9th day
	gm.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1	240	120	1200	§				
2	210	140	210	45	17.5	6.5	6	6
3	250	240	330	42	18	7.5	6	7
4	200	150	330	180	80	10	6	7
5	210	100	400	§				
6	295	240	§					
7	240	180	§					
Mother	6000	21	21	8	7	6	6	11

\* Approximately 1 hour after birth.

† On the 1st day Pups 2, 3, and 4 received 2 mg. of synkayvite intravenously and 1.5 mg. orally.

‡ On the 8th day, 60 mg. of dicumarol were given intravenously to the mother. Pup 2 was removed from the mother and was not allowed to nurse.

§ Died of hemorrhage.

|| 26 mg. of dicumarol were given orally during the last 4 days of gestation.

A small amount of dicumarol appears in the milk, since the prothrombin is diminished after suckling when the mother is fed dicumarol. This is demonstrated by results recorded in Tables II and III. The effect on the prothrombin is not striking and probably of no practical significance but is interesting since it shows that a small amount of the drug passes into the milk. Field (6) has recorded similar observations.

The sensitivity of the new-born pup to dicumarol can also be demonstrated by administering it directly. The effect of injecting 5 mg. per kilo of body weight into 20 day-old pups is recorded in Table IV. It will be observed that the fall of prothrombin is more precipitous and the depression much more prolonged than in adult dogs. In 24 hours the prothrombin



dropped from 100 to 7 to 10 per cent and in 48 hours to about 1 per cent and remained at this low level for 3 days, whereas in the mature dog, the lowest concentration of prothrombin was 5 per cent and a marked improvement appeared on the 4th day.

The greater susceptibility of the new-born pup to dicumarol is probably due to the rapid consumption of prothrombin by the organism. If it is assumed that dicumarol acts by blocking the enzymatic mechanism that synthesizes prothrombin, presumably by replacing vitamin K, the production of prothrombin begins to drop as soon as dicumarol reaches the liver and enters the cells responsible for the synthesis. The more prothrombin consumed, the faster is the blocking of the mechanism, and therefore the more precipitous the fall of prothrombin and the more pronounced the depression of the level.

TABLE IV

*Effect of Dicumarol on Prothrombin Time of Young Pups (20 Days Old)*

5 mg. of dicumarol per kilo of body weight were given intravenously.

Pup No.	Weight	Prothrombin time							
		0 day	1st day	2nd day	3rd day	4th day	5th day	7th day	10th day
	gm.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1	710	6	15	40	45	45	23	11	6
2	830	6	17	51	50	42	31	13	6
3	780	6	16	39	46	*			
4	885	6	18	46	45	53	19	9	6
5	600	6	18	50	35	62	16	11	6
6	739	6	17	50	55	65	31	15	6
Adult dog	6000	6	11	17	21	10	9	6	

\* Died of internal hemorrhage.

In spite of the low prothrombin level observed in these pups, only a few died of hemorrhage. In all but one that died the cause was massive intra-abdominal hemorrhage. It was not possible to find the point of bleeding. The fatal bleeding observed in Pup 3 of Table IV was presumably due to trauma. One of the pups of that litter accidentally fell from a laboratory desk to the floor. Unfortunately no record was made immediately, but on the basis of memory it appeared to have been Pup 3. Animals as well as man often may have a low prothrombin level for a considerable period of time but may not show any tendency to abnormal bleeding as long as they are protected from injury.

The results recorded in this paper have clinical significance. Since the new-born baby even normally tends to have a marked hypoprothrombinemia, it seems logical to surmise that dicumarol would further drastically

reduce the level. The administration of dicumarol to a pregnant woman can therefore be considered to be strictly contraindicated. It is probably quite safe, however, to give a nursing mother dicumarol, since the amount secreted into the milk is too small to affect significantly the prothrombin of the baby.

#### SUMMARY

1. The prothrombin level of pups is reduced at birth but becomes normal within the first few days of life.
2. The pups born of a mother fed dicumarol show a much greater reduction of the prothrombin concentration of the blood than that of the maternal organism.
3. Pups are more susceptible to dicumarol than are adult dogs.
4. The results of this study indicate that the use of dicumarol in a pregnant woman is strictly contraindicated.

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# A TRACER STUDY OF IRON METABOLISM WITH RADIOACTIVE IRON

## I. METHODS: ABSORPTION AND EXCRETION OF IRON

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The metabolism of iron is one of the most important subjects in the field of mineral metabolism. There have been numerous investigations of its problems, but until recently they have been seriously limited by the methods of study available. The production of radioactive isotopes of iron (1, 2) provided a new research tool which has proved of great value in the investigation of this field. Iron labeled with radioactive isotope may be distinguished from the iron originally present in the body, and so the fate of a given dose of radioiron<sup>1</sup> may be determined with some precision.

Previous work with radioiron in the rat (3) and in dogs (4) and humans (5) was handicapped by the low specific activity of the preparations of the radioactive isotope,  $\text{Fe}^{59}$ , which were hitherto available. This necessitated the use of massive doses of iron beyond the normal physiological limits.

In the present work, use was made of the radioactive isotope  $\text{Fe}^{55}$ , which is prepared in the cyclotron by bombardment of manganese with deuterons.<sup>2</sup> Since the only contamination with inert iron is that occasioned by traces of iron in the manganese probe, this isotope may be prepared with a very high specific activity. This made possible the use of very small doses (0.05 mg.) approaching tracer levels. These tracer doses were administered to rats by stomach tube or by injection, and studies were made of the absorption, excretion, storage, utilization, and distribution of iron under certain experimental conditions.

### Methods

*Preparation of Radioactive Iron*—The radioactive isotope  $\text{Fe}^{55}$  was prepared by deuteron bombardment ( $d, 2n$ ) of a manganese probe in the cyclotron.<sup>2</sup> The  $\text{Fe}^{55}$  produced decays by K electron capture with a half life

\* The material of this paper was taken from a thesis submitted by D. H. Copp to the Graduate Division of the University of California in partial fulfillment of the requirement of the degree of Doctor of Philosophy, June, 1943.

<sup>1</sup> Iron labeled by its content of radioactive isotope will be referred to as radioiron, or by the symbol  $\text{Fe}^*$ .

<sup>2</sup> Kamen, M. D., personal communication.

of approximately 4 years. It emits a soft  $\gamma$ -ray and very soft conversion electrons.

Scrapings from the manganese probe were dissolved in 6 M HCl and diluted to 1 M HCl. The copper present was precipitated with hydrogen sulfide and removed. Small amounts of non-radioactive Mn, Co, Zn, and phosphate were added as carriers to assist in removing any radioactive isotopes of these elements which might be present. A few mg. of iron were also added before the first precipitation, since only traces of iron were present in the probe. The iron was then precipitated from a chilled solution in 1 M HCl with a chilled 6 per cent aqueous solution of cupferron (6). This compound precipitates iron in the presence of dilute acid, leaving in solution the Mn, Co, Zn, and phosphate which might be radioactive contaminants of the target. The cupferron precipitate was ashed and dissolved in acid. After repeating this procedure four times, no radioactive contaminants could be detected in the filtrate, and the identity of the  $\text{Fe}^{55}$  was further confirmed by the characteristic absorption of its weak radiation.

The  $\text{Fe}^*$  was prepared for administration in neutral isotonic saline as ferric citrate, or iron ammonium citrate. The solution assayed 0.05 mg. of Fe per ml., with a specific activity of approximately 1 to 5 microcuries per mg. The dose administered was 1 ml. per rat.

*Dietary Regimens*—Rats were rendered iron-deficient by a modification of the procedure of Harris (7). They were weaned at 3 weeks to a diet of powdered whole milk supplemented with 5 mg. of thiamine chloride, 5 mg. of pyridoxine, and 50 mg. of calcium pantothenate per kilo. The diet assayed 5 parts per million of copper,<sup>3</sup> so that it could not be considered deficient in this element. This may account for the absence of the usual signs of copper deficiency in these animals. They were kept in glass cages with perforated aluminum floors, and were supplied with redistilled water *ad libitum*. When the radioactive iron was administered 5 weeks later, the level of blood hemoglobin was less than half that of the control animals.

Rats weaned to the regular stock colony diet served as controls. Both groups were about 2 months old and still rapidly growing when the dose of  $\text{Fe}^*$  was given. Later, experiments were conducted on 6 month-old adult female rats which had been reared and maintained on the stock diet, and which had ceased growing.

*Viviperfusion*—Because of the very high concentration of iron in the hemoglobin of blood, it is important that the tissues be completely freed of blood before iron analyses are carried out. This is most effectively accomplished by viviperfusion, a procedure whereby the living animal is

<sup>3</sup> Copper was assayed by the dithizone method through the kindness of Dr. D. I. Arnon, Division of Plant Nutrition, University of California, Berkeley.

perfused with a solution approximating the composition of blood serum until the blood is almost completely replaced by this fluid.

Whipple (8) first stressed the value of viviperfusion in obtaining reliable analyses of iron in tissues, and described a technique for dogs. A method for use in the rat is given by Austoni, Rabinovitch, and Greenberg (9). A simplification of this latter method was developed for the present investigation. It may easily be carried out on any small animal and should prove of value whenever it is desirable to obtain tissues free from blood.

The essential apparatus consists of a burette filled with perfusion fluid warmed to 50°. A modified Tyrode's solution of the following percentage, composition may be used: NaCl 0.80, KCl 0.02, CaCl<sub>2</sub> 0.02, MgCl<sub>2</sub> 0.01 NaH<sub>2</sub>PO<sub>4</sub> 0.005, NaHCO<sub>3</sub> 0.10, and glucose 0.10. The last two components are added just before use.

The rat is anesthetized with nembutal (4 mg. per 100 gm. of body weight) injected intraperitoneally. It is tied to an operating board, the abdomen is opened, and the inferior vena cava is exposed. A large bore needle (18 gage) is inserted in the vein, and a blood sample withdrawn for determination of blood hemoglobin, total iron, and radioiron. With the needle in place, the syringe is detached and its place is taken by the barrel of a tuberculin syringe connected by flexible rubber tubing to the tip of the burette, so that warm perfusion fluid flows directly through the needle into the vein. The needle is ligated in place and the abdomen is closed with clamps. The jugular veins in the neck are next dissected out and opened so that the perfusion fluid flowing into the inferior vena cava may flush out the right auricle and escape with the blood from the jugulars. The veins in the legs are also opened. The animals usually survive from 20 to 40 minutes. At death, the lungs should be white, the liver a light fawn color, and the heart should be full of clear fluid. Presence of clear fluid in the portal vein is a good indication of complete perfusion. Occasionally, when this fluid is tinged with blood, it may be necessary to perfuse the liver directly through the portal vein to clear it completely of blood.

The organs were dissected out with bright chrome scissors and wet-ashed. The blood sample was corrected for total blood weight from the figures given by Donaldson for the rat (10). Muscle stripped from the hind limbs was similarly corrected. Red bone marrow samples (50 to 100 mg.) were obtained by splitting the femurs and tibias and scraping the marrow cavity.

The perfused tissues of a series of normal and iron-deficient rats were determined. The results are given in Table I. These values show reasonable agreement with those obtained by Austoni, Rabinovitch, and Greenberg for rats (9) and by Bogniard and Whipple for dogs (11).

*Ashing Procedure*—To avoid the considerable loss of iron which may occur when tissue samples containing chloride are dry-ashed (12), tissue

and excreta samples were wet-ashed in small Erlenmeyer flasks by adding successive portions of concentrated  $\text{HNO}_3$  and cooking to dryness on a hot-plate. 5 to 50 ml. of  $\text{HNO}_3$  were usually adequate for complete digestion. A few drops of superoxol aided the process. The total iron in each sample was corrected by subtracting the iron in the acid used for ashing.

The great bulk of the carcass made wet ashing inconvenient. It was accordingly dry-ashed at dull red heat to minimize iron loss. Despite this precaution, the recovery of  $\text{Fe}^*$  from the carcass was often less than that calculated from its blood and muscle content, suggesting a loss of volatile iron during ashing.

The ash was dissolved in 1 M  $\text{HCl}$ , made to volume, and aliquots were taken for determination of total iron and radioactive iron.

TABLE I  
*Iron Content\* of Tissues of Normal and Iron-Deficient Animals Freed of Blood by Viviperfusion*

Tissues	Normal rats†		Iron-depleted rats†	
	No. of samples		No. of samples	
Body weight, gm.....	24	125 $\pm$ 8	24	114 $\pm$ 14
Blood Hb, gm. per 100 ml.....	24	14.1 $\pm$ 1.1	24	6.4 $\pm$ 1.3
Blood*.....	24	44.5 $\pm$ 3.2	24	20.1 $\pm$ 4.2
Liver*.....	19	8.3 $\pm$ 4.0	19	2.8 $\pm$ 0.8
Spleen*.....	23	32.7 $\pm$ 14.0	24	12.2 $\pm$ 3.8
Bone marrow*.....	20	21.3 $\pm$ 7.2	12	12.0 $\pm$ 3.5
Skeletal muscle*.....	14	1.4 $\pm$ 0.4	22	0.9 $\pm$ 0.3

\* Iron content is expressed as mg. of Fe per 100 gm. of fresh tissue.

† The figures given are the mean values  $\pm$  the standard deviation.

*Determination of Total Iron.*—The total iron was determined on an aliquot from each sample by the *o*-phenanthroline colorimetric method described by Saywell and Cunningham (13) and critically reviewed by Fortune and Mellon (14). Colorimetric readings were made with a Klett-Summerson photoelectric colorimeter with green Filter 54. This instrument was also used for the determination of hemoglobin by the acid hematin method.

*Determination of Radioactive Iron.*—So soft is the radiation of  $\text{Fe}^{55}$  that the count on a sample is reduced to half by as little as 10 mg. of ash per sq. cm. This difficulty was overcome by electroplating the iron in a thin layer for which the absorption of radiation was nil. The method used was a simplified adaptation of that described by Hahn, Bale, and Balfour (15) and by Ross and Chapin (16). The apparatus is shown in Fig. 1. Seamless tin ointment capsules serve as electrolytic cells. The capsules are held in place on a rack with steel paper clips. These are connected to the

negative terminal of a source of 110 volts d.c., so that the metal bottom of the ointment capsule becomes the cathode on which the iron is deposited. A platinum anode in the center of the capsule is connected through a 15 watt lamp resistance to the other terminal.

The iron sample, or an aliquot, is pipetted into the capsule, and 1 ml. of plating solution is added. This solution, adapted from that used by Hahn *et al.* (15) is made up in distilled water with the following percentage composition: ferric citrate 1.1 ( $\approx$  2 mg. of Fe per ml.), sodium citrate 25.0, ammonium chloride 12.5. The plating is carried out for 5 hours, an additional 1 ml. of plating solution being added as carrier at half time. The solution in the capsule at the end of the process should give little or no iron color with thiocyanate. The capsules are rinsed, dried and the

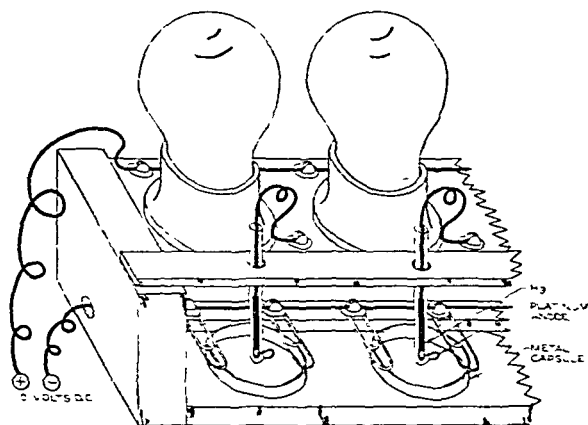


FIG. 1. Apparatus for electroplating of radioactive iron

radioiron determined. Since the radiations from  $\text{Fe}^{55}$  are too weak to register on the usual metal or glass Geiger counter tubes, use was made of the thin mica window counter tube described by Copp and Greenberg (17).

In ten trials in which standard amounts of  $\text{Fe}^*$  were added to solutions of tissue ash, the recovery by the above method was  $99.7 \pm 1.8$  per cent. When considerable quantities of calcium were present (as in the ash from carcass or feces) precipitation of calcium salts interfered with the plating. To avoid this, the calcium was first precipitated with potassium oxalate at pH 4, and the precipitate was filtered off. When this was done, the recovery was comparable to that with other tissues.

The recovery of the administered  $\text{Fe}^*$  was determined by adding the values for the individual tissues, excreta, and residual carcass, and was

found to be 80 to 90 per cent of the administered dose. Some of the reasons for failure to obtain complete recovery have been mentioned by Hahn *et al.* (4). Much of the difficulty may be due to loss of iron during dry ashing of the carcass.

### EXPERIMENTAL

*Excretion of Parenterally Administered Radioiron*—It has been difficult to obtain conclusive evidence on the excretion of iron from conventional chemical balance studies. However, by injecting labeled radioiron, it is possible to determine exactly how much of the administered iron is excreted. A dose of 0.05 mg. of  $\text{Fe}^*$ , equivalent to less than 1 per cent of the total body iron, was given to rats on stock diet by intravenous or intraperitoneal injection. The animals were sacrificed at 12, 24, 48, and 96 hours. The average excretion is shown in Table II. It may be seen that

TABLE II  
*Excretion\* of Parenterally Administered Radioiron*

	Time following injection			
	12 hrs.	24 hrs.	48 hrs.	96 hrs.
	8 rats	6 rats	5 rats	6 rats
Urine....	0.3	0.7	0.2	1.1
Feces...	0.1	0.4	0.1	0.4
Total..	0.4	1.1	0.3	1.5

\* The figures given are the mean values expressed as per cent of the total dose of  $\text{Fe}^*$ .

the excretion of  $\text{Fe}^*$  in both urine and feces is small and quite variable. The lack of any significant excretion of even this tracer dose of  $\text{Fe}^*$  confirms the observations of Hahn *et al.* on dogs (18). This was in striking contrast to the behavior of cobalt and manganese, elements which are adjacent to iron in the periodic table. When tracer doses of the radioactive isotopes of these elements were injected parenterally, a large part of the dose was excreted within the first 2 days, cobalt appearing principally in the urine (19), while manganese was eliminated chiefly in the feces (20).

The excretion of  $\text{Fe}^*$  in the bile of rats with a biliary fistula and artificial gallbladder has been reported in a previous publication (21). Only traces of  $\text{Fe}^*$  (0.1 per cent) were excreted in the first 48 hours, in contrast to the significant amount of  $\text{Co}^*$  (2 to 4 per cent) and the large proportion of  $\text{Mn}^*$  (24 to 40 per cent) which appeared in the bile.

The actual excretion of  $\text{Fe}^*$  into the lumen of the intestinal tract was investigated in two groups of rats which were given the radioiron by sub-



cutaneous or intraperitoneal injection. After viviperfusion, the small and large intestines were removed separately and the contents were carefully washed out. Both intestines and contents were analyzed for  $\text{Fe}^*$  separately, and the results are presented in Table III. From these values, it appears that, while considerable amounts of  $\text{Fe}^*$  are taken up by the tissues of the intestine, only traces find their way into the lumen.

*Absorption of Radioiron from Intestinal Tract*—Since only traces of iron are normally excreted, it is evident that the iron in the body must be regulated by absorption. Here, too, labeled radioiron provides a means of determining exactly how much of a given dose is absorbed. 1 ml. doses containing 0.05 mg. of  $\text{Fe}^*$  were administered by stomach tube to normal and iron-deficient growing 2 month-old rats. This dose, which is much less than the normal daily intake of iron in food, may be expected to follow the same path of metabolism as the normal dietary iron. Such small doses lie within physiological limits, and avoid the possible complications of

TABLE III  
*Excretion\* of Radioiron into Lumen of Intestinal Tract*

	Time following injection			
	12 hrs	24 hrs.	48 hrs.	96 hrs.
	3 rats			4 rats
Small intestine (washed out)	2.5	1.6	1.7	1.6
Contents of small intestine	0.2	0.2	0.1	0.4
Large intestine (washed out)	0.8	0.5	0.8	0.8
Contents of large intestine	0.2	0.6	0.5	0.7

\* The figures given are the mean values expressed as per cent of the total dose of  $\text{Fe}^*$ . The radioiron was administered by intraperitoneal or subcutaneous injection.

massive iron concentrations. After administration of the  $\text{Fe}^*$ , the rats were sacrificed at various time intervals up to 4 days. The stomach, small intestine, large intestine, and feces, including their contents, were analyzed for  $\text{Fe}^*$ . The values, plotted against time, are shown in Fig. 2.

In the normal control rats, the administered  $\text{Fe}^*$  passed rapidly along the intestinal tract. The stomach emptied quickly, and within 3 hours a considerable part of the dose had passed through the small intestine to reach the large bowel. Significant amounts had appeared in the feces by 12 hours, and almost all of the unabsorbed iron had been excreted within the first 24 hours.

In the iron-deficient rats, the  $\text{Fe}^*$  passed along the intestinal tract at a much slower rate, as was observed by Austoni and Greenberg (3). The poor intestinal tone observed in these anemic animals may account for the

delay. Very little  $\text{Fe}^*$  had reached the large bowel at 3 hours, so that the large amount of  $\text{Fe}^*$  which had already appeared in the body at this time must have been absorbed from the stomach or small intestine. The most striking delay, however, occurred in the large intestine. Significant

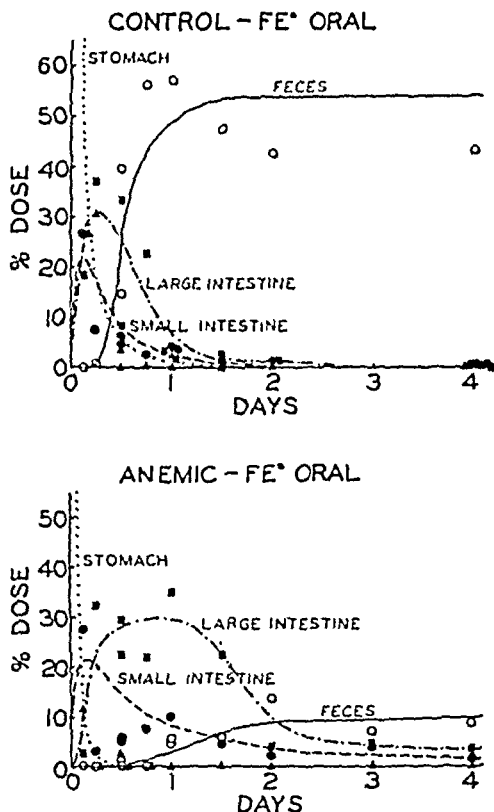


FIG. 2. Passage of the dose of radioiron along the gastrointestinal tract. Dose of 0.05 mg. of  $\text{Fe}^*$  administered by stomach tube. The control rats were 2 months old, and had been reared and maintained on the stock colony diet. The average weight of the group was  $124 \pm 7$  gm., and the mean value for the blood hemoglobin was  $14.4 \pm 1.1$  gm. per 100 ml. The anemic rats were weaned at 3 weeks to a milk diet, and were used when 2 months old. The average weight of these animals was  $112 \pm 11$  gm., and the mean value for the blood hemoglobin was  $5.8 \pm 1.1$  gm. per 100 ml. Stomach  $\blacktriangle$  ..... , small intestine  $\bullet$  — — —, large intestine  $\blacksquare$  — · — ·, feces  $\circ$  — — —.

amounts of  $\text{Fe}^*$  did not reach the feces for 24 hours, and a considerable part of the dose was retained for almost 2 days. Since only a third of the  $\text{Fe}^*$  in the large intestine was ultimately excreted in the feces, it would appear that considerable absorption may take place in this organ.

The iron-deficient rats absorbed over nine-tenths of the tracer dose of  $\text{Fe}^*$ , indicating a very efficient utilization of dietary iron. While the slow rate at which the dose passed along the intestinal tract may have facilitated this efficient utilization by allowing more time for absorption, it was not the sole factor, since the iron-depleted animals absorbed the  $\text{Fe}^*$  much more rapidly from the very beginning.

The control animals on the other hand absorbed less than a third of the dose. This confirms the observations on dogs (4) and humans (5) for massive doses of  $\text{Fe}^*$ .

#### DISCUSSION

Since only insignificant amounts of iron are normally excreted, the iron balance in the animal must be regulated by control of absorption. Iron-depleted rats absorbed  $\text{Fe}^*$  much more efficiently than did the controls, even though the latter were still growing actively and therefore required some iron for the synthesis of new hemoglobin. Hahn *et al.* (4) observed that absorption of large doses of radioiron by normal adult dogs was negligible, while iron-depleted animals absorbed considerable amounts.

The difference in absorption was not due to anemia *per se*, since it does not occur in cases of untreated pernicious anemia (5) nor in acute anemia in the dog (22). In the latter case, however, when the level of the blood hemoglobin had been restored to normal at the expense of depleted iron stores, absorption increased 5 to 10 times. The weight of evidence supports the thesis of Whipple *et al.* (23) that "absorption of iron is dependent on the need of the body for iron."

The most efficient absorption of  $\text{Fe}^*$  was obtained when minute doses of  $\text{Fe}^*$  were fed to rats on a milk diet very low in iron. Since the concentration of iron in the lumen of the gut is very low in these animals, it seems highly improbable that the process is one of simple diffusion dependent on differences in ionic concentration as has been suggested by McCance and Widdowson (24). Indeed the evidence indicates that the absorption of iron may be a specific process involving the intestinal mucosa. The relative uptake of  $\text{Fe}^*$  by the gut and the rapid rate of turnover reported in Paper II of this series lend further support to this view. Hahn *et al.* (22) observed a "mucosal block" a few hours after feeding iron (but not after injection of colloidal iron) which prevented further absorption. They suggested that this might be due to saturation of the mechanism involved. The probable nature of this mechanism has been indicated by Granick (25) who observed the appearance of the iron containing protein ferritin in the wall of the intestine of growing guinea pigs following iron feedings. This presumably reaches equilibrium with the other iron reserves of the body, and its state of depletion or saturation, reflecting that

of the general body stores, determines the absorption of iron from the intestine.

On continued feeding of iron, he found ferritin in the walls of the small and large intestine, and in smaller amounts in the stomach and even the cecum. This adds some support to our indirect evidence for absorption from the small intestine and large intestine, and for the demonstration by Hahn *et al.* (22) of absorption from gastric, duodenal, and jejunal pouches. It would appear that iron may be absorbed from any part of the gastrointestinal tract.

#### SUMMARY

1. Iron labeled with the radioactive isotope  $\text{Fe}^{55}$  was prepared with a high specific activity compared to that usually obtained with  $\text{Fe}^{59}$ . For this reason, small tracer doses (0.05 mg. of  $\text{Fe}^*$ ) could be used for administration to rats. Because of the soft character of the radiations from  $\text{Fe}^{55}$ , the iron in the samples analyzed was first electroplated, and the radioactivity was then measured with a thin mica window Geiger counter tube.

2. Experiments were conducted on normal 2 month-old rats, and on anemic rats which had been depleted of iron by rearing them on a diet of powdered milk.

3. A simplified method of viviperfusion was developed to free the tissues and organs of blood.

4. No significant excretion of  $\text{Fe}^*$  was observed in the bile, urine, or feces following parenteral administration of the tracer dose.

5. Iron-depleted rats absorbed over 90 per cent of the dose of  $\text{Fe}^*$ , while the normal growing rats absorbed less than one-third. Absorption apparently took place in both the small and large intestine.

6. There was a relatively high uptake of  $\text{Fe}^*$  by the intestinal wall, although only traces were excreted into the lumen.

7. Some factors concerned in the absorption of iron are discussed, and a possible mechanism is suggested.

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# A TRACER STUDY OF IRON METABOLISM WITH RADIOACTIVE IRON

## II. INTERNAL METABOLISM

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The metabolism of iron in the body is of great interest. In the past, conventional chemical methods have yielded little satisfactory information on this subject because of the wide variations in the iron content of normal tissues. Even when massive doses of iron were used, it was difficult to obtain gross differences in the tissue iron which could be considered significant.

The radioactive isotopes now available have proved ideal tools for studies of internal metabolism, since it is possible to follow the exact fate in the body of an element or substance so labeled. Unfortunately the radioactive isotope of iron commonly employed,  $\text{Fe}^{59}$ , has only been available in preparations of such low specific activity that very large doses had to be used. Since such massive doses are not very suitable for metabolic studies, attention was until recently focused on the absorption and excretion of radioiron.<sup>1</sup>

The production of  $\text{Fe}^{55}$  with a high specific activity made possible the use of small doses of radioiron which were much more suited to the study of internal metabolism. With such tracer doses, the following problems were investigated: (a) the fate of orally administered and injected radioiron in normal and iron-depleted young rats, (b) the effect of various routes of administration on the storage of radioiron, and (c) the relationship between utilization and storage in adult rats, in which hemopoiesis had been stimulated by blood loss or cobalt treatment.

### Methods

The analytical and biological methods used in this investigation have been described in Paper I of this series (1). A dose of 1 ml. containing 0.05 mg. of  $\text{Fe}^*$  as neutral citrate in physiological saline was administered

\* The material of this paper was taken from a thesis submitted by D. H. Copp to the Graduate Division of the University of California in partial fulfillment of the requirement of the degree of Doctor of Philosophy, June, 1943.

<sup>1</sup> Iron labeled by its content of radioactive isotope will be referred to as radioiron, or by the symbol  $\text{Fe}^*$ .

to each rat. The animals were sacrificed by viviperfusion at time intervals up to 4 days. Tissues and excreta were wet-ashed and analyzed for total iron and radioiron. The results are presented graphically in Figs. 1 to 12. The graphs are similar, so that they may be compared directly. In the left-hand graph of each pair, the per cent of the administered dose of  $\text{Fe}^*$  in liver and blood is plotted against time. Since practically all of the radioiron in the blood at 24 hours is in the form of hemoglobin (2), the per cent of  $\text{Fe}^*$  in blood at this time gives a measure of the iron utilization for hemoglobin synthesis over this period. In the right-hand graph, the tissue concentration of  $\text{Fe}^*$  (expressed as the per cent of the administered dose per gm. of fresh tissue) is plotted against time. These curves show the specific uptake of  $\text{Fe}^*$  by blood, liver, spleen, and bone marrow, and the changes in distribution with time.

#### EXPERIMENTAL

*Fate of Orally Administered  $\text{Fe}^*$  in Normal and Iron-Depleted Rats*—Rats weaned to a powdered milk diet at 3 weeks were depleted of iron as described in Paper I (1). Both these anemic rats and control rats which had been kept on the stock colony diet were given the  $\text{Fe}^*$  by stomach tube at 2 months of age. The results are shown in Figs. 1 and 2.

In the normal control animals, the limited amount of radioiron absorbed was rapidly built into hemoglobin. This was reflected in the active turnover of  $\text{Fe}^*$  in bone marrow. Only small amounts were deposited in the liver and spleen.

The iron-depleted rats absorbed a much larger proportion of the administered dose, and this radioiron was very rapidly built into new hemoglobin. The turnover of  $\text{Fe}^*$  in bone marrow was most active, but only limited amounts appeared in liver and spleen. There was no apparent interference with hemoglobin production or retention of iron in the liver such as might indicate copper deficiency (3). This was not surprising in view of the relatively high copper content (5 parts per million) of the milk diet used.

*Fate of Intraperitoneally Injected  $\text{Fe}^*$  in Normal and Iron-Depleted Rats*—The fate of intraperitoneally injected  $\text{Fe}^*$  was studied in similar groups of normal and iron-depleted rats. In addition, one group of iron-depleted rats was given a simultaneous injection of 0.1 mg. of Cu as copper sulfate. The results are given in Figs. 3 to 5.

A comparison with the curves for the orally administered  $\text{Fe}^*$  reveals a striking difference in the uptake of  $\text{Fe}^*$  by the liver. Within 3 hours after injection, the liver had taken up almost half of the dose in the control animals, and over a quarter of the dose in the iron-depleted animals. The 24 hour level of radioiron in the blood was several times greater in the iron-depleted rats than in the controls, indicating a much more rapid



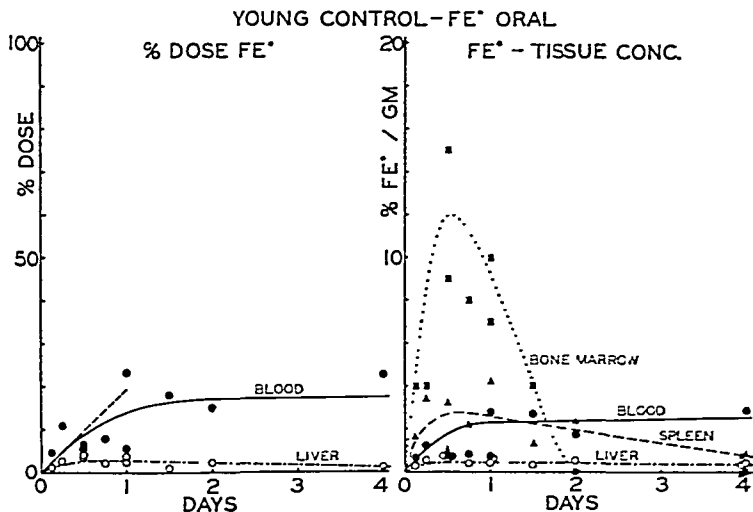


FIG. 1. Fate of orally administered radioiron in normal rats. The average body weight of the group was  $124 \pm 7$  gm., and the mean value for the blood hemoglobin was  $14.4 \pm 1.1$  gm. per 100 ml. Blood  $\bullet$ —, liver  $\circ$ —, spleen  $\blacktriangle$ —, bone marrow  $\blacksquare$ .

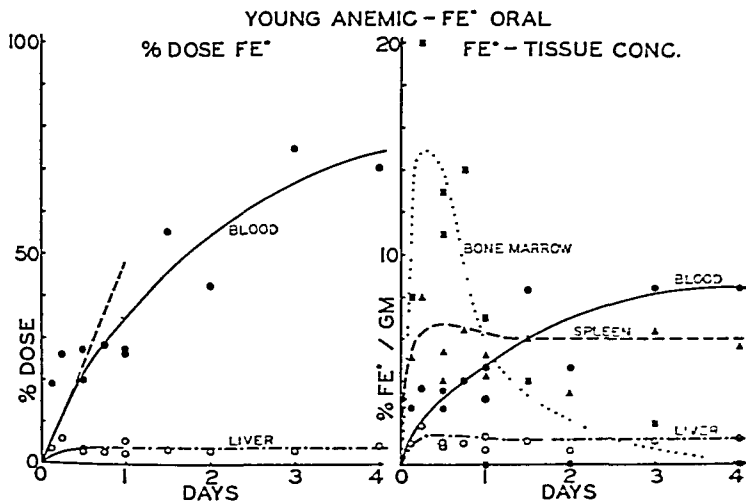


FIG. 2. Fate of orally administered radioiron in anemic rats. The average body weight of the group was  $112 \pm 11$  gm., and the mean value for the blood hemoglobin was  $5.8 \pm 1.1$  gm. per 100 ml. The symbols are as in Fig. 1.

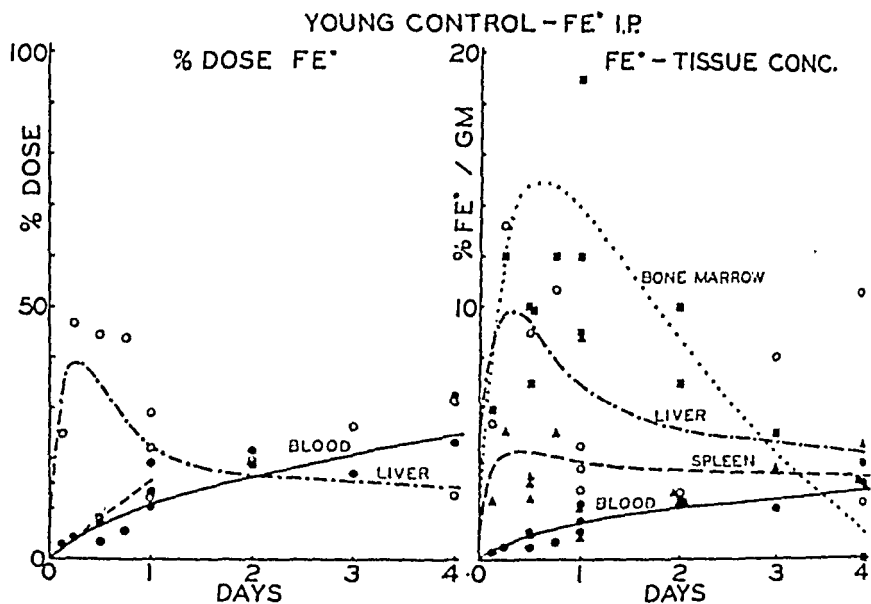


FIG. 3. Fate of intraperitoneally injected radioiron in normal rats. The average body weight of the group was  $128 \pm 7$  gm., and the mean value for the blood hemoglobin was  $14.1 \pm 1.1$  gm. per 100 ml. The symbols are as in Fig. 1.

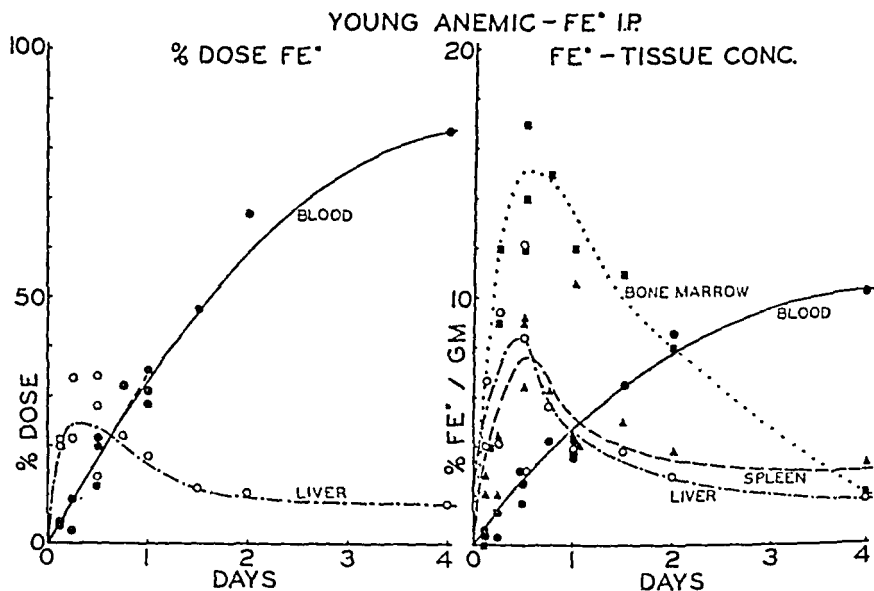


FIG. 4. Fate of intraperitoneally injected radioiron in anemic rats. The average body weight of the group was  $116 \pm 12$  gm., and the mean value for the blood hemoglobin was  $6.8 \pm 1.1$  gm. per 100 ml. The symbols are as in Fig. 1.

utilization of  $\text{Fe}^*$  in hemoglobin synthesis by the former. Administration of copper increased this effect by some 50 per cent and greatly diminished the storage of  $\text{Fe}^*$  in the liver. This corresponds with the general effect of copper noted by Elvehjem and Sherman (3) in their anemic rats, despite the fact that the gross symptoms of copper deficiency were not evident in our animals.

Despite the great bulk of muscle, only 2 to 6 per cent of the dose was taken up by this tissue in either group, with no significant difference be-

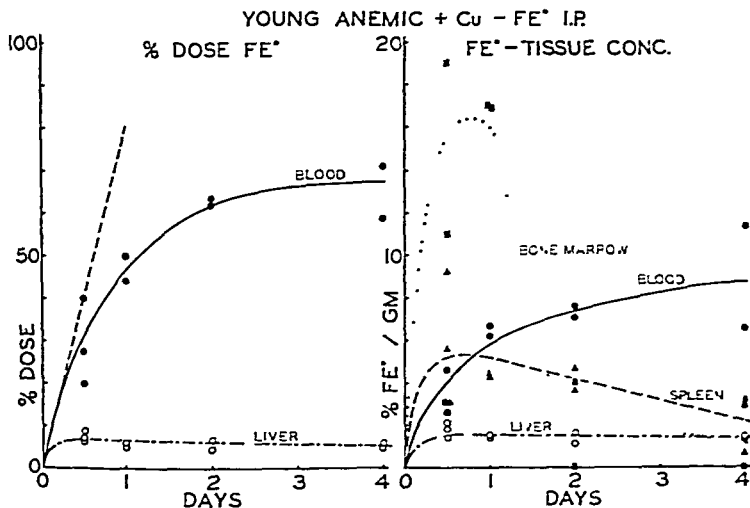


FIG. 5. Fate of intraperitoneally injected radioiron in anemic rats when copper was administered simultaneously. The average body weight of the group was  $116 \pm 10$  gm., and the mean value for the blood hemoglobin was  $7.5 \pm 1.1$  gm. per 100 ml. The symbols are as in Fig. 1.

tween the normal and anemic animals. The muscle iron appeared to be rather stable, and there was no evidence for storage in this tissue.

There was a rapid turnover of  $\text{Fe}^*$  in bone marrow, which was much greater in the iron-depleted animals than in the controls, particularly when copper was also administered. Although no figures are available for bone marrow weight in the rat, estimates for dogs (4) and rabbits (5) indicate that this organ may represent about 2 per cent of the total body weight. Assuming a similar ratio in the rat, the tissue concentration of  $\text{Fe}^*$  in bone marrow would indicate that soon after the dose of  $\text{Fe}^*$  is administered as much as one-quarter to one-half may be taken up by this organ. The half

period of retention of the  $\text{Fe}^*$  in bone marrow appears to be only 1 or 2 days, indicating a rapid rate of turnover associated with formation of new hemoglobin.

*Specific Activity and Tissue Turnover*—Values for specific activity are extremely valuable in determining the metabolic activity of iron in various tissues. They are expressed as the per cent of the total iron in a tissue which has come from the administered dose of  $\text{Fe}^*$ . Values for specific activity in blood, bone marrow, liver, spleen, and intestine are shown plotted against time in Figs. 6 and 7.

### SPECIFIC ACTIVITY — $\text{Fe}^*/\text{Fe} \times 100\%$

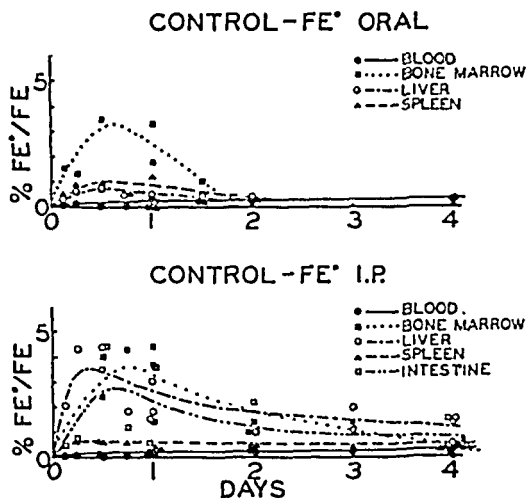


FIG. 6. Specific activity of the radioiron in the tissues of normal rats

When the radioiron was administered orally, the specific activity in liver and spleen at first exceeded that in blood, indicating a possible initial storage function. The subsequent decline in the  $\text{Fe}^*$  in these organs as the  $\text{Fe}^*$  level in the blood increased suggested that some of this stored  $\text{Fe}^*$  may have been utilized for hemoglobin synthesis. The highest specific activity was found in bone marrow, as might be expected from its function.

When the radioiron was injected intraperitoneally, the most striking feature was the remarkable uptake of  $\text{Fe}^*$  by the liver. In the normal rats, the peak specific activity in liver equaled that in bone marrow, and far exceeded that in spleen or blood. The effect was much more pronounced in the iron-depleted rats, where as much as 15 to 20 per cent of the total iron in the depleted organ was  $\text{Fe}^*$  derived from the injected dose. In

contrast, when copper was also administered, there was only a small transitory rise in liver  $\text{Fe}^*$ . This liver storage was only temporary, and within 24 hours over half of the  $\text{Fe}^*$  in the liver had disappeared. Since this was accompanied by a corresponding rise in  $\text{Fe}^*$  in blood, it is probable that this  $\text{Fe}^*$  had been made available for hemoglobin synthesis.

A second interesting observation was the rapid turnover of  $\text{Fe}^*$  in the small intestine of both normal and iron-depleted rats. The peak specific activity following injection of radioiron was almost as high as in the case of bone marrow, although the rate of turnover did not appear to be as great. This rapid metabolism of radioiron in the small intestine may be associated

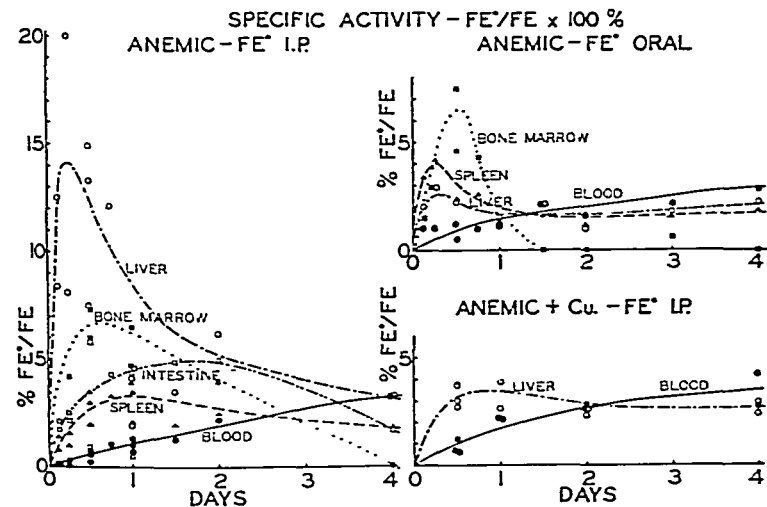


Fig. 7. Specific activity of the radioiron in the tissues of anemic rats. The symbols are as in Fig. 6.

with the important rôle suggested for this organ in regulating the absorption of iron (6).

*Effect of Route of Administration of Radioiron*—The striking difference in uptake of  $\text{Fe}^*$  by the liver following oral and intraperitoneal administration suggested further work on the effect of various routes of administration. Adult female rats (6 to 8 months old) maintained on the stock colony diet were used for these experiments. A dose of 1 ml. containing 0.05 mg. of  $\text{Fe}^*$  was given by intravenous, subcutaneous, or intraperitoneal injection. The results are shown in Figs. 8 to 10. Almost half of the radioiron injected intravenously (Fig. 9) was found in the liver at 24 hours,

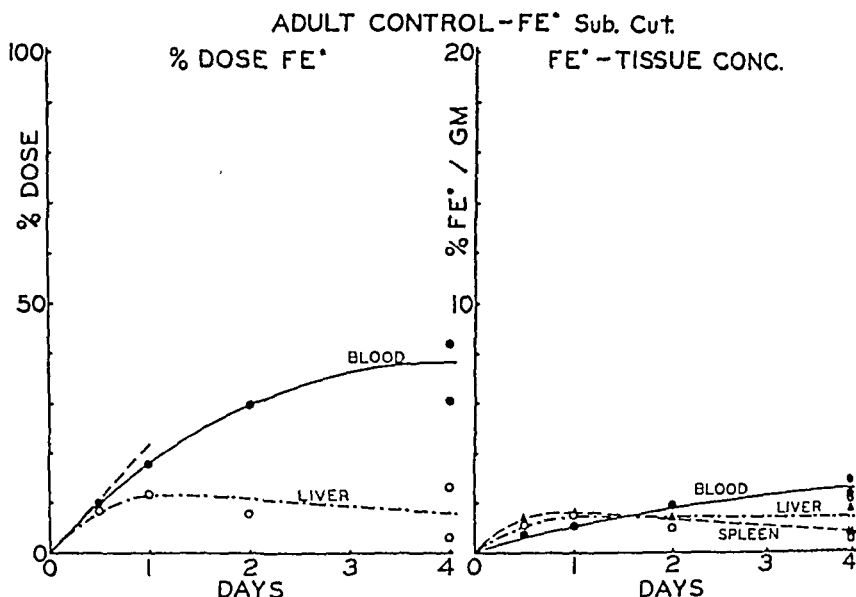


FIG. 8. Fate of subcutaneously injected radioiron in normal adult rats. The average body weight of the group was  $213 \pm 11$  gm., and the mean value for the blood hemoglobin was  $16.3 \pm 0.7$  gm. per 100 ml. Blood  $\bullet$ —, liver  $\circ$ —, spleen  $\blacktriangle$ —.

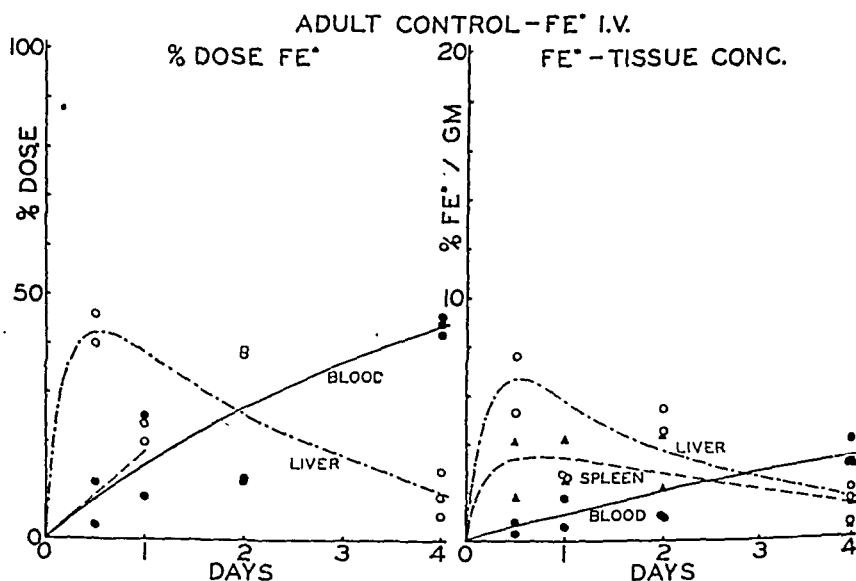


FIG. 9. Fate of intravenously injected radioiron in normal adult rats. The animals used were adult female rats which were 6 to 8 months old, and had been reared and maintained on the stock diet. The symbols are as in Fig. 8.

in good agreement with the observations of Granick and Hahn in dogs (7). Following intraperitoneal injection in young (Fig. 3) and adult (Fig. 10) rats, storage in liver at 24 hours amounted to 30 to 40 per cent of the administered dose, as compared to around 10 per cent following subcutaneous injection (Fig. 8), and 3 per cent following oral administration (Fig. 1). There appears to be a direct correlation between the rate at which the  $\text{Fe}^*$  is introduced into the blood stream and the proportion of the dose which is stored initially in the liver. This may in turn be related to the level of serum iron. Moore *et al.* (8) have shown that the intravenous injection of iron causes a tremendous increase in the level of serum iron, while

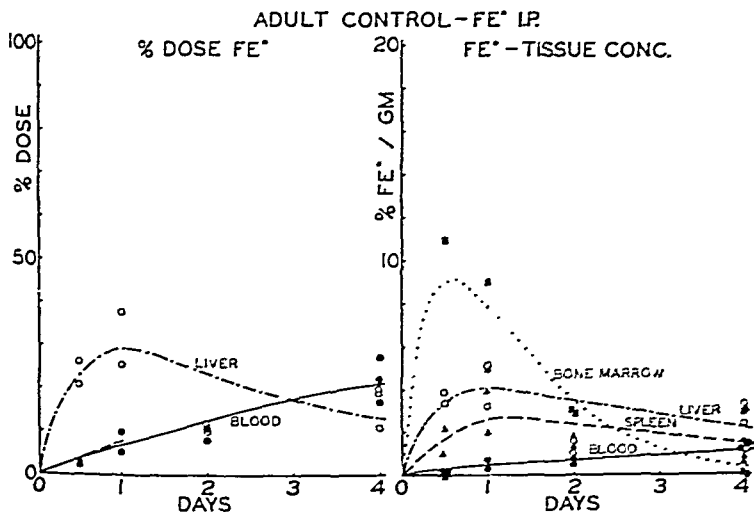


FIG. 10. Fate of intraperitoneally injected radioiron in normal adult rats. The average body weight of the group was  $222 \pm 15$  gm., and the mean value for the blood hemoglobin was  $16.7 \pm 0.9$  gm. per 100 ml. The symbols are as in Fig. 1.

the same amount given orally produces only a small rise. Although only 0.05 mg. of  $\text{Fe}^*$  was injected into these animals, it would still be sufficient to cause a 5-fold increase in serum iron if given by intravenous or intraperitoneal injection. This suggests that the liver may have an important rôle in regulating the level of serum iron by temporarily storing this excess.

*Effect of Bone Marrow Stimulation by Cobalt or Blood Loss*—The effect of bone marrow stimulation was studied to determine its influence on liver storage of iron. Adult female rats (6 to 8 months old) reared and maintained on the stock colony diet were used. The radioiron (0.075 mg.) was given by intraperitoneal injection. In the first group (Fig. 11), the

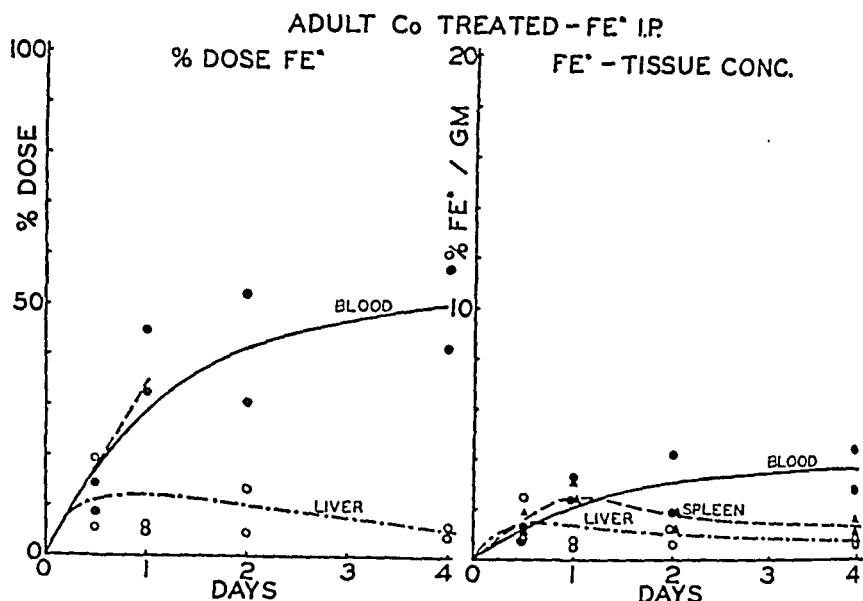


FIG. 11. Fate of intraperitoneally injected radioiron in adult rats receiving polycythemic doses of cobalt. The average weight of the group when sacrificed was  $201 \pm 14$  gm., with an average loss in weight of 25 gm. over the 2 week period when cobalt was being administered. The mean value for the blood hemoglobin was  $18.3 \pm 0.8$  gm. per 100 ml. The symbols are as in Fig. 8.

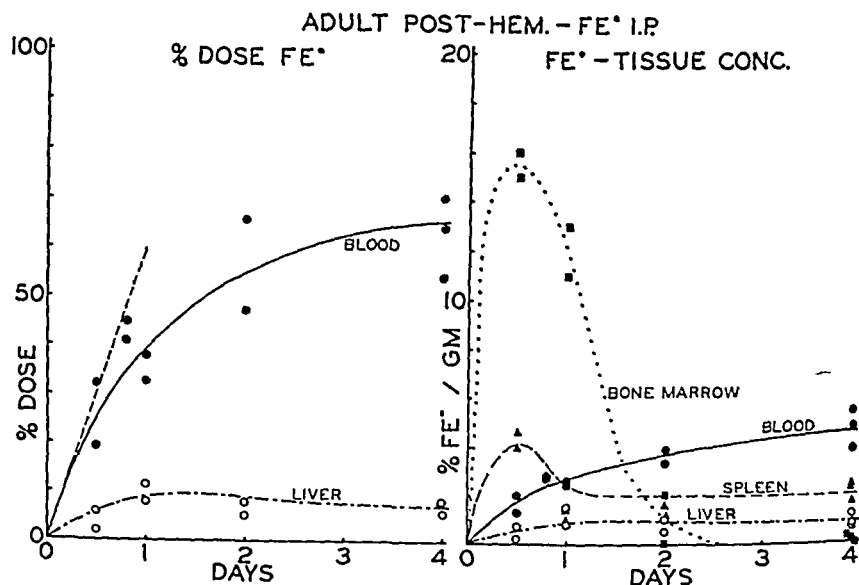


FIG. 12. Fate of intraperitoneally injected radioiron in adult rats following severe blood loss. The average body weight of the group was  $216 \pm 18$  gm., and the mean value for the blood hemoglobin was  $14.5 \pm 0.8$  gm. per 100 ml. The symbols are as in Fig. 1.



bone marrow was stimulated by administration of polycythemic doses of cobalt (9) (0.1 mg. of Co as cobalt acetate injected intraperitoneally) every other day for 2 weeks. In the second group (Fig. 12), approximately one-third of the blood volume was removed by heart puncture to simulate the effects of severe hemorrhage. When the  $\text{Fe}^*$  was injected 5 days later, the blood hemoglobin of these animals had almost returned to normal. The resulting curves should be compared with those in Fig. 10 for normal rats given  $\text{Fe}^*$  by intraperitoneal injection.

Both groups of rats showed definite evidence of increased  $\text{Fe}^*$  utilization in hemoglobin production. The level of  $\text{Fe}^*$  in the blood at 24 hours was increased 4-fold in the group given cobalt, and 6-fold in those recovering from blood loss. Of particular interest was the relatively small amount

TABLE I  
*Summary of Essential Data from Graphs*

Condition of experimental rats	Route by which radioiron was administered	Fig. No.	Radioiron in blood at 24 hrs.	Peak storage of radioiron in liver
			<i>per cent of total dose</i>	<i>per cent of total dose</i>
Young control	Stomach tube	1	14	3
" anemic	" "	2	35	4
" control	Intraperitoneally	3	11	39
" anemic	" "	4	33	25
" " + injection of copper	" "	5	47	7
Adult control	Subcutaneously	8	18	11
" "	Intravenously	9	15	42
" "	Intraperitoneally	10	7	30
" given cobalt treatment	" "	11	29	12
Adult following severe blood loss	" "	12	39	10

of  $\text{Fe}^*$  deposited in the livers of both groups, despite the fact that the dose was injected intraperitoneally. The rapid utilization of  $\text{Fe}^*$  by the stimulated bone marrow may have left little excess to be stored in the liver.

#### DISCUSSION

These studies on the turnover of radioiron in various tissues have provided new data concerning the behavior of iron in the body. Some of the pertinent points are summarized for comparison in Table I.

The important function of the bone marrow in the internal metabolism of iron was substantiated by the marked uptake of  $\text{Fe}^*$  by this organ, and the rapid rate of turnover. The half period of retention of the  $\text{Fe}^*$  in bone marrow was of the order of 1 or 2 days. With the 24 hour uptake of  $\text{Fe}^*$

by the blood as an index of the utilization of  $\text{Fe}^*$  in hemoglobin synthesis over this period, stimulated marrow activity was demonstrated in anemic iron-depleted rats (particularly with copper), in adults receiving polycythemic doses of cobalt, and in adult rats recovering from blood loss.

As might have been predicted from the relatively high copper content of the milk diet used, none of the usual signs of copper deficiency were evident in the anemic rats when the  $\text{Fe}^*$  was administered orally. There was active utilization of the dose of  $\text{Fe}^*$ , and no abnormal storage of  $\text{Fe}^*$  in the liver. However, when the radioiron was injected intraperitoneally, simultaneous administration of copper increased the rate of  $\text{Fe}^*$  utilization for hemoglobin production, and greatly decreased the storage of  $\text{Fe}^*$  in the liver. This suggests a latent copper deficiency, and provides a very sensitive means for demonstrating it.

The chief organ for iron storage appears to be liver, with the spleen playing a very secondary rôle, and the muscle being relatively unimportant. The importance of liver in iron storage, first stressed by Jacobi (10) and Schmidt (11), has been amply confirmed by the excellent work of Granick and Hahn (7) using radioiron. The latter demonstrated a tremendous uptake of  $\text{Fe}^*$  in the liver of dogs following intravenous injection. They were able to show that most of this stored  $\text{Fe}^*$  was in the iron-rich protein, ferritin.

The route of administration was found to have a profound effect on the amount of  $\text{Fe}^*$  stored in the liver. This was probably correlated with changes in the level of serum iron. Intravenous injection, with its resultant high rise in serum iron, was associated with marked liver storage, while oral or subcutaneous administration, which gives only a small increase in serum iron, resulted in very limited storage in that organ. The amount of  $\text{Fe}^*$  in the liver was also much less when the  $\text{Fe}^*$  was being actively utilized in hemoglobin synthesis by bone marrow which had been stimulated by cobalt or by blood loss.

#### SUMMARY

1. Tracer experiments with radioiron (0.05 mg.) in rats substantiated the important rôle of bone marrow in iron metabolism. Following injection, a large part of the dose of radioiron was taken up by this organ and used in hemoglobin synthesis. The turnover in the marrow was rapid, with a half period of only 1 or 2 days.

2. The radioiron provided a very sensitive method of demonstrating iron utilization in hemoglobin production. Increased bone marrow activity with respect to this function was demonstrated in iron-depleted rats, growing rats, in adults given polycythemic doses of cobalt, and in adult rats following blood loss.

3. None of the usual signs of copper deficiency were evident when the radioiron was administered orally to the anemic iron-depleted rats reared on a milk diet, owing to the considerable copper content of the milk. However, when the radioiron was injected, simultaneous administration of copper increased the rate of  $\text{Fe}^*$  utilization and diminished the storage in liver, thus providing a very sensitive test of latent copper deficiency.

4. The liver was found to be the chief site of iron storage, the amount of radioiron stored being greatest when the dose was injected intravenously, and least following oral administration. Liver storage was also greatly decreased when the radioiron was being actively utilized in hemoglobin synthesis by stimulated bone marrow.

5. A rapid rate of turnover in the small intestine was observed, which may be associated with its rôle in regulating absorption.

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# FURTHER STUDIES OF ORGANIC FACTORS REQUIRED FOR PREVENTION OF ANEMIA IN CHICKS\*

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The results of studies in several laboratories (1-3) have shown that folic acid is required for the prevention of anemia in the chick. The anti-anemic effect of  $\beta$ -pyracin lactone when administered together with the fermentation *Lactobacillus casei* factor (LCF), the folic acid conjugate of Hutchings, Stokstad, Bohonos, and Slobodkin (4), has been demonstrated by Scott, Norris, Heuser, and Bruce (5). In earlier studies reported by Scott, Norris, Heuser, Bruce, Coover, Bellamy, and Gunsalus (6)  $\alpha$ -pyracin lactone was found to prevent anemia in chicks fed a diet containing factor S concentrate from yeast, described by Schumacher, Heuser, and Norris (7). Later it was found that the supply of factor S concentrate used in this study contained a larger amount of folic acid than usual, chiefly in the conjugate form.

Studies with factor S concentrate conducted in this laboratory by Hill (8) and by Scott and associates (5, 6) have consistently shown a slight but positive effect upon hemoglobin formation by this preparation, which at times could not be accounted for in terms of either folic acid or pyracin content of the preparation.

Since pyracin had been found to be active in the prevention of anemia in chicks when supplied together with folic acid conjugate, and since factor S appeared to have some effect upon hemoglobin formation in the chick, an investigation was undertaken to determine (a) whether pyracin is required for the prevention of anemia in the presence of free folic acid, and (b) whether factor S contains an antianemic factor not identical with folic acid or pyracin. The results of the investigation are presented in this report.

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## EXPERIMENTAL

White Leghorn male chicks were used in the experimental work. The chicks were fed a purified diet described earlier (5), except that phthalylsulfathiazole was substituted for the succinylsulfathiazole. The method of handling the diet was the same as that described in the earlier report (5).

Since crystalline folic acid was not available in sufficient quantities for use in this work, a liver folic acid concentrate which contained only free folic acid was used. This concentrate contained 1250  $\gamma$  of folic acid per gm. by *Streptococcus faecalis* assay and 1300  $\gamma$  per gm. by *Lactobacillus casei*, a synthetic folic acid being used as the standard. It was fed at a level which provided 100  $\gamma$  of folic acid per 100 gm. of diet,  $\beta$ -pyracin at 100  $\gamma$  per gm., and factor S concentrate at a level in the diet equivalent to 5 per cent yeast. These three supplements were included individually and in all possible combinations.

The quantities of folic acid and  $\beta$ -pyracin supplied in this study were in excess of the requirements. Robertson, Daniel, Farmer, and Norris<sup>1</sup> have shown that the quantitative requirement of the chick for folic acid, when fed the diet used in this work, is 40 to 50  $\gamma$  per 100 gm. of diet for optimum growth, 30 to 40  $\gamma$  per 100 gm. for optimum hemoglobin formation, and 20 to 30  $\gamma$  per 100 gm. for the prevention of mortality. Scott and associates (5) have shown 50  $\gamma$  of pyracin to be sufficient for the prevention of anemia when supplied together with 50  $\gamma$  of LCF per 100 gm. of diet.

The results presented in Table I show that in the presence of the folic acid concentrate the inclusion of  $\beta$ -pyracin had no effect upon hemoglobin level or growth, contrary to previous findings at this laboratory when conjugates were used as the source of folic acid. After 4 weeks on the experimental diets, however, the hemoglobin level of the chicks receiving the folic acid concentrate had not reached a maximum but was 1.3 gm. per 100 ml. of blood lower than the hemoglobin level of those chicks receiving a commercial chick diet. Factor S concentrate, when fed alone or in combination with  $\beta$ -pyracin, had little effect upon either chick growth or hemoglobin response, but, when it was supplied together with the folic acid concentrate, it maintained the hemoglobin level at values approximately equal to those obtained with the commercial diet.

A statistical analysis of the hemoglobin values by "Student's" method for unpaired data was conducted to determine whether the increase in hemoglobin was biologically important. The  $t$  value obtained for the significance of the difference was 8.52. With approximately 60 values

<sup>1</sup> Robertson, E. I., Daniel, L. J., Farmer, F. A., and Norris, L. C., unpublished data.

per group, the odds are 9999:1 when  $t$  equals 5.1. The results showed, therefore, that the increase in hemoglobin level of approximately 1 gm. per 100 ml. of blood was highly significant.

Microbiological assays of the factor S concentrate used in these studies showed that, even after incubation with liver enzymes, the amount of folic acid in this preparation was low. It contributed less than 5  $\gamma$  of folic acid per 100 gm. of diet when fed at the level equivalent to 5 per cent yeast. Since, in the lots receiving factor S concentrate alone, or with pyracin, approximately 75 per cent of the chicks died in 4 weeks, further evidence is provided that the factor S concentrate contained little folic acid. In view of the fact that the factor S concentrate was found to be a poor source of folic acid, and because the liver folic acid concentrate provided almost

TABLE I  
*Results Showing Antianemic Activity of Factor S*

Supplement	No of chicks surviving at 4 wks.	Average chick weight at 4 wks	Average Hb at 4 wks.
		gms.	gm. per 100 ml
None.....	6 (15)*	94	2.3
Factor S.....	4 (15)	93	5.0
$\beta$ -Pyracin.....	8 (30)	103	3.5
Factor S + $\beta$ -pyracin....	7 (30)	112	3.7
Folic acid..	15 (15)	232	8.5
" " + $\beta$ -pyracin ..	45 (45)	276	8.6
Factor S + folic acid ...	14 (15)	232	9.7
" " + " " + $\beta$ -pyracin.	44 (45)	237	9.5
Commercial chick diet ...	15 (15)	291	9.8

\* The figures in parentheses indicate the number of chicks started.

double the amount of this vitamin shown to be required by Robertson and associates,<sup>1</sup> the effect of the factor S concentrate upon hemoglobin formation cannot be explained in terms of additional folic acid and, therefore, must have been produced by some other factor present in the factor S concentrate.

Since the folic acid was supplied to the chicks in this study in the form of a liver concentrate, the possibility existed that the liver concentrate contained pyracin and, therefore, additional pyracin was not required for prevention of anemia. In view of this a study was undertaken to ascertain whether or not  $\beta$ -pyracin was present in the concentrate.

Huff and Perlzweig (9) have pointed out that the lactone of 4-pyridoxic acid ( $\beta$ -pyracin lactone), when irradiated with ultraviolet light, emits a strong blue fluorescence, the intensity of which varies with the pH of the

lactone solution. They have also reported that the acid form of this compound can be converted into the lactone by boiling in a mineral acid. Accordingly, 0.1 gm. of liver folic acid concentrate was refluxed in 50 ml. of 0.1 N HCl for 15 minutes. The solution was neutralized with sodium hydroxide and its fluorescence intensity determined at a level of 20  $\gamma$  of the concentrate per ml. over a pH range from 6.5 to 10.0. The fluorescence intensity of this solution, which served as the control, is shown by Curve A in Fig. 1.

Another 0.1 gm. sample of the concentrate was refluxed in 50 ml. of 1.0 N hydrochloric acid for 2 hours, cooled, and neutralized. Curve B in Fig. 1 shows the fluorescence intensity obtained with this solution of hydrolyzed folic acid concentrate at a level of 20  $\gamma$  of the concentrate per ml. over the

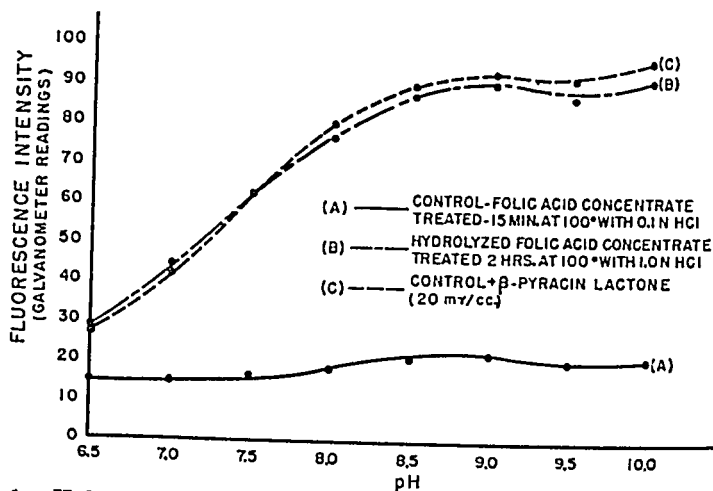


FIG. 1. pH-fluorescence curves showing presence of  $\beta$ -pyracin in liver folic acid concentrate.

same pH range. Curve C in Fig. 1 shows the pH-fluorescence curve produced by the addition of 20 millimicrograms of pure  $\beta$ -pyracin lactone per ml. of control solution. The two latter curves are almost superimposed. This is evidence that the liver folic acid concentrate contains pyracin. The amount of  $\beta$ -pyracin, calculated from these results, was approximately 1 mg. per gm. of folic acid concentrate. Thus the folic acid concentrate contributed about 90  $\gamma$  of  $\beta$ -pyracin per 100 gm. of diet at the level fed to the chicks.

By means of ether extraction at pH 6.5 of an acid-hydrolyzed solution of 30 gm. of the folic acid concentrate, followed by adsorption and elution from Super Filtrol and subsequent purification by sublimation under re-



duced pressure, 5 mg. of a crystalline substance were obtained. The pH-fluorescence curve of the crystals was identical with that of pure synthetic  $\beta$ -pyracin lactone at the same concentration.

Since these findings strongly indicate that pyracin was present in the liver concentrate, no conclusions could be drawn from this study as to whether or not pyracin is required for prevention of anemia in the presence of free folic acid.

Therefore, when synthetic folic acid became available later, a second experiment was undertaken. The chicks were fed the purified diet supplemented with factor S concentrate equivalent to 5 per cent yeast. Synthetic folic acid and  $\beta$ -pyracin were supplied singly and in combination at levels of 100  $\gamma$  per 100 gm. of diet. The liver folic acid concentrate was also included in this experiment at a level to provide 100  $\gamma$  of folic acid per

TABLE II

*Results Showing Prevention of Anemia by Synthetic Folic Acid in Absence of Pyracin*

Supplement	No. of chicks surviving at 4 wks.*	Average chick weight at 4 wks.	Average Hb at 4 wks.
		gm.	gm. per 100 ml.
Factor S.....	0		
" " + $\beta$ -pyracin	4	103	2.4
" " + synthetic folic acid	17	252	9.5
" " + " " " " + $\beta$ -pyracin .....	17	241	9.6
Factor S + liver folic acid concentrate	16	241	9.5

\* Each lot was started with seventeen white Leghorn chicks of mixed sex.

100 gm. of diet, so that the results could be compared with those obtained in the previous experiment. The results of this study are presented in Table II. They show that the synthetic folic acid promoted a hemoglobin level which was not increased by the addition of pyracin to the diet.

#### DISCUSSION

From the data presented in this report, it is evident that factor S concentrate contains an antianemic factor which is effective in raising the hemoglobin level in chicks above that obtained with folic acid.

Hill (8) has shown that factor S is not removed from solution by adsorption with activated charcoal (Darco G-60) at pH 3.0. In the course of his studies with factors R and S of Schumacher and associates (7), Hill found that he could obtain the factor R fraction free of factor S by treating the former concentrate with charcoal, thus leaving factor S in the fil-

trate. This adsorbate of factor R contained most of the folic acid present in the original factor R concentrate. After treatment of factor S concentrate with charcoal, he found that the non-adsorbable fraction contained factor S, but was free of folic acid. When the adsorbate of factor R was supplied to chicks fed a purified diet, the average hemoglobin level in the chicks at 5 weeks was 6.8 gm. per 100 ml. Adding the folic acid-free factor S concentrate alone to the diet failed to promote any increase in hemoglobin level over that of the control chicks. The hemoglobin levels for these lots averaged 3.0 and 3.5 gm. per 100 ml., respectively. However, when the adsorbate of factor R and the folic acid-free factor S concentrate were fed together, the average hemoglobin level in the chicks at 5 weeks was 8.5 gm. per 100 ml. of blood.

Campbell, Brown, and Emmett (1) have demonstrated that crystalline vitamin B<sub>c</sub> (folic acid), when added to a purified diet very similar in composition to the one used in the present investigation, is almost, but not quite, as effective as a normal ration in preventing anemia in chicks up to 4 weeks of age. Vitamin B<sub>c</sub> at a level of 100  $\gamma$  per 100 gm. of diet promoted a hemoglobin level of 7.6 gm. per 100 ml. 4 times this amount of vitamin B<sub>c</sub> resulted in a hemoglobin level of only 7.7 gm. per 100 ml., while the chicks receiving a normal broiler ration had an average hemoglobin of 8.7 gm. per 100 ml. of blood. The hemoglobin results which they reported are, therefore, strikingly similar to the ones reported here for the liver folic acid concentrate. The fact that the normal broiler ration promoted a hemoglobin level approximately 1 gm. per 100 ml. of blood higher than the plateau response obtained with vitamin B<sub>c</sub> may mean that the normal broiler ration contained factor S, which was lacking in their purified diet.

As a result of studies on vitamins B<sub>10</sub> and B<sub>11</sub> and related substances, Briggs, Luckey, Elvehjem, and Hart (10) have suggested the existence of a new hemoglobin factor. They found that liver fractions low in vitamin B<sub>c</sub> (folic acid) activity raised the hemoglobin values in chicks, and that other fractions rich in either vitamin B<sub>10</sub> or B<sub>11</sub> did not completely prevent anemia. They showed that  $\alpha$ -pyracin lactone was not identical with the suggested new antianemic factor. It is possible that this antianemic activity was due to factor S, as indicated in this report.

Daniel, Scott, Norris, and Heuser (11) have shown that pyracin is necessary in order to convert LCF into folic acid and thus make it available to *Streptococcus faecalis*. From their results, they concluded that the action of pyracin in promoting an increased production of folic acid from LCF is caused either by conjugation with LCF to form folic acid, or by the fact that pyracin functions in an enzyme system required for the breakdown of LCF and the setting free of folic acid. It has been announced by Jukes

and Stokstad<sup>2</sup> that LCF appears to be a folic acid conjugate. Therefore, the latter explanation of Daniel and associates is probably the correct one.

The studies presented in this report have shown that pyracin is not required for hematopoiesis in the chick when the diet contains free folic acid. On the other hand, since pyracin is required with certain folic acid conjugates for the prevention of anemia in chicks, further evidence is provided that it enters into an enzyme system required for the breakdown of folic acid conjugates. This suggests the possibility that  $\beta$ -pyracin is the prosthetic group of an enzyme.

#### SUMMARY

The hemoglobin level in chicks has been significantly increased by the addition of factor S from dried brewers' yeast to a diet containing adequate amounts of folic acid and  $\beta$ -pyracin. This demonstrates that factor S possesses antianemic activity.

Pyracin has been found not to be required for the prevention of anemia in chicks when the diet contains free folic acid. Since  $\beta$ -pyracin is necessary with certain folic acid conjugates for the prevention of anemia in chicks, further evidence is provided that it functions in an enzyme system required for the breakdown of folic acid conjugates and setting free of folic acid.

We wish to thank Dr. L. E. Arnow of Sharp and Dohme, Inc., Glenolden, Pennsylvania, for the phthalylsulfathiazole; Dr. T. H. Jukes of the Lederle Laboratories, Inc., Pearl River, New York, for the liver folic acid concentrate and the synthetic folic acid; Dr. Karl Folkers of Merck and Co., Inc., Rahway, New Jersey, for the  $\beta$ -pyracin; and Mr. E. A. Webb of Anheuser-Busch, Inc., St. Louis, Missouri, for the dried brewers' yeast.

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# THE OCCURRENCE OF HYDROXYPYRUVIC ACID IN BIOLOGICAL SYSTEMS\*

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(Received for publication, March 29, 1946)

In the course of studies conducted in this laboratory on the breakdown of hydroxyamino acids in enzyme systems, a new 2,4-dinitrophenylhydrazone which fulfilled the analytical requirements of a derivative of *hydroxypyruvic acid*,  $\text{HOCH}_2\cdot\text{CO}\cdot\text{COOH}$ , was isolated, following the deamination of *dl*-serine by rat kidney slices. The discovery of this simplest hydroxy-keto acid, produced under nearly physiological conditions, appeared of sufficient interest to warrant the studies presented in this and the following paper (1).

*dl*-Serine is known to be deaminated oxidatively by mammalian kidney, although at a slower rate than the most readily attacked substrates such as *dl*-alanine or *dl*-methionine (2-5). On the other hand, the very rapid deamination of *dl*-serine by a number of microorganisms (6-9) was recently shown in this laboratory to proceed, by the way of a dehydration under anaerobic conditions, to pyruvic acid (10, 11). *dl*-Threonine undergoes a similar sequence of reactions (11). The same mechanism appears to prevail in the deamination of *dl*-serine by extracts of mammalian liver (11, 12) and muscle (12).

Although 2,4-dinitrophenylhydrazones of  $\alpha$ -keto acids have been isolated following the oxidative deamination of several amino acids by rat kidney slices in the presence of arsenite (2, 13), there is no report of a similar experiment with *dl*-serine. When rat kidney slices were permitted to act on *dl*-serine in 0.05 M bicarbonate-Ringer's solution, hydroxypyruvic acid could invariably be isolated as the dinitrophenylhydrazone mentioned previously. These preparations and those secured from synthetic hydroxypyruvic acid (1) proved to be identical.

There is still some doubt as to the enzyme system responsible for this deamination. Under conditions affording hydroxypyruvic acid dinitrophenylhydrazone from *dl*-serine it has not been possible to obtain a hydra-

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† This report is from a dissertation submitted by David B. Sprinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

zone, following the action of rat kidney slices on *l*-serine or on *dl*-threonine. A quantitative comparison between *l*- and *dl*-serine showed that 90 per cent of *l*-serine remained unattacked, only negligible amounts of ammonia being evolved, whereas *dl*-serine lost 36 per cent of its initial concentration and gave rise to 22 per cent of ammonia.<sup>1</sup> The deaminating effect of rat kidney slices on *dl*-serine, therefore, appears to be limited to the *d* variety. It is nevertheless not possible to attribute it to *d*-amino acid oxidase, since purified preparations of this enzyme, in contrast to kidney slices or crude extracts (2-5, 15), attack *dl*-serine either very slowly (15, 16) or not at all (17).

*d*-Serine has been reported to produce an often fatal injury in rats, characterized by necrosis of the kidney tubules (18), when administered by stomach tube in conjunction with a diet deficient in protein and the B vitamins (19, 20). Since it seemed possible that hydroxypyruvic acid might be the metabolic intermediate responsible for the serine injury, the action of *dl*-serine in producing the reported typical kidney lesions in rats was compared with that of synthetic hydroxypyruvic acid (1). Whereas there was no difficulty in confirming previous findings with *dl*-serine (18), hydroxypyruvic acid, under similar experimental conditions, was without effect.

It is not yet possible to assign a definite metabolic function to hydroxypyruvic acid. In isolated enzyme systems *l*-serine does not appear to undergo transamination (21, 22) or deamination, though *l*-phosphoserine has been claimed to take part in transamination reactions ((23, 24); however, compare (22)). The resulting phosphoric acid ester of hydroxypyruvic acid could then give rise to the hydroxyketo acid by phosphatase action.

#### EXPERIMENTAL

*Formation of Hydroxypyruvic Acid from dl-Serine by Rat Kidney Slices*—The buffer solution used consisted of 100 cc. of 0.154 M NaCl, 4 cc. of 0.154 M KCl, 3 cc. of 0.11 M CaCl<sub>2</sub>, and 20 cc. of 0.31 M NaHCO<sub>3</sub> which had been saturated with CO<sub>2</sub>. Before use this mixture was equilibrated with O<sub>2</sub> containing 5 per cent (by volume) of CO<sub>2</sub>.

To 50 cc. of the buffer solution in a Krebs vessel (2) were added 500 mg. of *dl*-serine (Merck), 0.08 cc. of 0.05 M NaAsO<sub>2</sub>, and 0.32 gm. (dry weight) of rat kidney slices. The vessel was placed in a thermostat at 38° and the CO<sub>2</sub>-O<sub>2</sub> mixture was aspirated through it for 15 minutes. The closed vessel was then shaken for 4 hours. The slices were removed, dried, and weighed. The addition of 300 mg. of 2,4-dinitrophenylhydrazine in 30 cc. of 2 N

<sup>1</sup> About 10 per cent of the *l*- or *dl*-serine remains unaccounted for either as ammonia or hydroxyamino acid. It is possible that the conversion of *l*-serine to glycine in the rat (14) is responsible for this observation.

HCl to the deproteinized (10 cc. of 30 per cent trichloroacetic acid) and filtered solution produced a precipitate which, after being stored in the refrigerator overnight, was filtered off and dried *in vacuo* over  $P_2O_5$ . The crude semicrystalline product, which weighed 75 mg., melted at 158–160°. After two crystallizations from ethyl acetate-ligroin (b.p. 60–90°), clusters of fine orange-colored needles were obtained, which melted at 162°. The mixture of this compound with a synthetic specimen of *hydroxypyruvic acid 2,4-dinitrophenylhydrazone* (1) melted at 159–161°. For analysis<sup>2</sup> the substance was thoroughly dried at 80° and 0.1 mm.

$C_9H_8O_7N_4$ .	Calculated.	C 38.0, H 2.8, N 19.7
284.2	Found.	" 37.9, " 2.9, " (Dumas) 19.7

This derivative of hydroxypyruvic acid could be isolated in all individual experiments when the conditions described here were maintained. No hydrazone could be obtained when Krebs' phosphate buffer (2) was substituted. This is in contrast to the behavior of *d*-amino acid oxidase (2). Since all buffers used contained arsenite, added in order to prevent the enzymatic breakdown of the formed keto acid, the difference in results may be attributed to the non-formation rather than to the disappearance of the reaction product.

In contrast to *dl*-serine, *l*-serine<sup>4</sup> failed to yield any comparable derivative, nor could the dinitrophenylhydrazone of  $\beta$ -hydroxy- $\alpha$ -ketobutyric acid be obtained, following the action of rat kidney slices on *dl*-threonine.

*Serine Balance and Ammonia Production Following Incubation of l- and dl-Serine with Rat Kidney Slices*—The trichloroacetic acid filtrate obtained in deamination experiments such as the one described above was neutralized with sodium bicarbonate. Ammonia was determined by aeration after the addition of a boric acid-sodium hydroxide buffer. Serine was determined by oxidation with sodium periodate and isolation of the formaldehyde dimedon derivative (25).

The incubation of 100 mg. (0.95 mM) of *l*-serine with 0.3 gm. (dry weight) of rat kidney slices, as previously described, gave a solution from which 0.02 milliequivalent of ammonia and 250 mg. (0.85 mM) of the formaldehyde dimedon derivative (m.p. 187–189°) were obtained.

The deamination of 500 mg. (4.76 mM) of *dl*-serine by 0.8 gm. (dry weight) of rat kidney slices yielded 1.04 milliequivalents of ammonia and 884 mg. (3.02 mM) of formaldehyde dimedon (m.p. 187–189°).

*Feeding Experiments with Sodium Hydroxypyruvate*—The 0.05 M hydroxy-

<sup>2</sup> The melting points, reported without correction, were determined with an electrically heated stage (Fisher-Johns).

<sup>3</sup> We are indebted to Mr. W. Saschek for the microanalyses.

<sup>4</sup> We are grateful to Dr. J. S. Fruton of Yale University for a specimen of *l*-serine.

pyruvate solutions (containing equivalent amounts of sodium bromide) were prepared daily by hydrolyzing 1.67 gm. (0.01 mole) of bromopyruvic acid with the required amount of 0.1 N NaOH, as is described in the following paper (1). Each batch was concentrated in a nitrogen atmosphere under reduced pressure to a volume of 30 cc. The freshly prepared solution was immediately administered by stomach tube in 3 cc. doses.

The *dl*-serine was similarly given in 3 cc. of water containing 0.001 mole of amino acid and of NaBr. The control group received 0.001 mole of NaBr in 3 cc. of water.

Three groups, each of five male rats, weighing 90 to 100 gm., were maintained on the experimental diet for 7 days previous to the administration of the test compounds. The diet (compare (18)) was composed of "Labco" vitamin-free casein 10 parts, dextrin 37, sucrose 37, Crisco 5, cod liver oil 5, Eimer and Amend Cellulation 2, and salt mixture (Smaco) 4. Water was supplied *ad libitum*. The animals showed practically no change in weight throughout this experiment. On the 8th, 9th, and 10th days *dl*-serine or sodium hydroxypyruvate was fed in the amounts above indicated. All animals showed symptoms of bromide intoxication. On the 11th day they were killed and the kidneys subjected to histological examination.<sup>5</sup> The animals that had received *dl*-serine exhibited kidney lesions essentially similar to those described by Morehead *et al.* (18). The typical injury was, however, not observed in the rats to which sodium hydroxypyruvate had been administered nor in the control group.

#### SUMMARY

Hydroxypyruvic acid 2,4-dinitrophenylhydrazone was isolated after the deamination by rat kidney slices of *dl*- but not of *l*-serine. The constitution of this derivative was established by comparison with a synthetic specimen. Parallel determinations of ammonia and unattacked hydroxyamino acid confirm the view that it is the *d* isomer that is mainly deaminated; *d*-amino acid oxidase, however, does not appear to be involved.

*dl*-Threonine did not give rise to a 2,4-dinitrophenylhydrazone under the same conditions.

Hydroxypyruvic acid was without effect in producing the typical kidney lesions caused by *d*-serine.

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<sup>5</sup> We should like to express our appreciation to Dr. H. C. Stoerk of the Department of Pathology for these examinations.



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# A STUDY OF $\beta$ -HYDROXY- $\alpha$ -KETO ACIDS\*

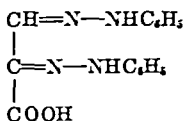
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The preparation of *hydroxypyruvic acid*,  $\text{CH}_2\text{OH}\cdot\text{CO}\cdot\text{COOH}$ , has often been claimed in the older literature, but the proofs of structure put forward have left much in doubt. The demonstration that this keto acid is a product of the enzymatic oxidative deamination of *dl*-serine (1) has prompted the detailed study of the hydroxyketo acids corresponding to serine and threonine, *i.e.* of hydroxypyruvic and  $\beta$ -hydroxy- $\alpha$ -ketobutyric acids.

Hydroxypyruvic acid was presumed by Will, in 1891 (2), to be present in the products of the decomposition of cellulose nitrate by alkali. The principal evidence consisted in the precipitation from the acidified reaction mixture of the lead salt of an acid that was not oxidized by bromine and yielded the osazone of mesoxalic acid semialdehyde (I). These



(I)

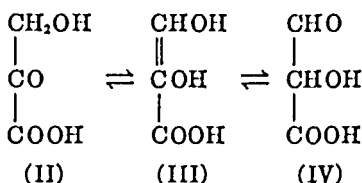
experiments were later confirmed (3) and descriptions of the isolation of what appears to be the same osazone can be found repeatedly in the literature; it was, for instance, obtained after the oxidation of glyceric acid with ferrous ion and hydrogen peroxide (4, 5) and from the products of the photochemical oxidation of aspartic (6) and tartaric (7) acids. Compound I also was isolated (8) after the treatment with phenylhydrazine of hiptagenic acid,  $\text{C}_3\text{H}_5\text{O}_4\text{N}$  (9), a product of the hydrolysis of two naturally occurring glucosides, hiptagin (9) and karakin (10). This acid is probably closely related to hydroxypyruvic acid, being an oximino or hydroxylamino derivative of this keto acid or of one of its tautomers.

The possibility of tautomerism between hydroxypyruvic acid (II),

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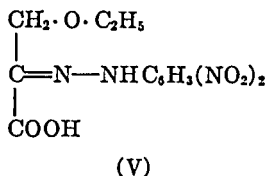
† This report is from a dissertation submitted by David B. Sprinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

dihydroxyacrylic acid (III), and tartronic acid semialdehyde (IV) is obvious. Because of the stability of the product formed by alkali from cellulose nitrate toward bromine and alkali, Will assigned to it structure II. Further support was supplied to this assumption later (11), although evidence favoring structure IV was also produced (12). Compound III is known in the form of certain derivatives, *e.g.* ethyl  $\beta$ -hydroxy- $\alpha$ -ethoxy-



acrylate,  $\text{HO}\cdot\text{CH}=\text{C}(\text{OC}_2\text{H}_5)-\text{COOC}_2\text{H}_5$  (13). Solutions of tartronic acid semialdehyde (IV) have been made available by the work of Fischer, Baer, and Nidécker (14).

A simple synthetic method for the preparation of solutions of  $\beta$ -hydroxy- $\alpha$ -keto acids was found in this laboratory. It consists in the careful hydrolysis of the corresponding  $\beta$ -bromo- $\alpha$ -keto acids. Hydroxypyruvic acid (II), obtained in this manner from bromopyruvic acid,  $\text{BrCH}_2\cdot\text{CO}\cdot\text{COOH}$ , gave an insoluble lead salt and readily reduced Benedict's solution. The results of the oxidation of freshly prepared solutions with periodic acid, to be discussed in the following paragraph, established beyond doubt that compound II actually had been obtained. In contrast to the products previously mentioned (2-5) this compound did not yield the osazone of mesoxalic acid semialdehyde (I). The hydroxypyruvic acid 2,4-dinitrophenylhydrazone, however, was obtained in a 90 per cent yield. The structure of this derivative was demonstrated by its reduction to *dl*-serine, apparently the first instance of the synthesis of an amino acid from a dinitrophenylhydrazone. Bromopyruvic acid itself gave a 2,4-dinitrophenylhydrazone which, with sodium hydroxide in aqueous ethyl

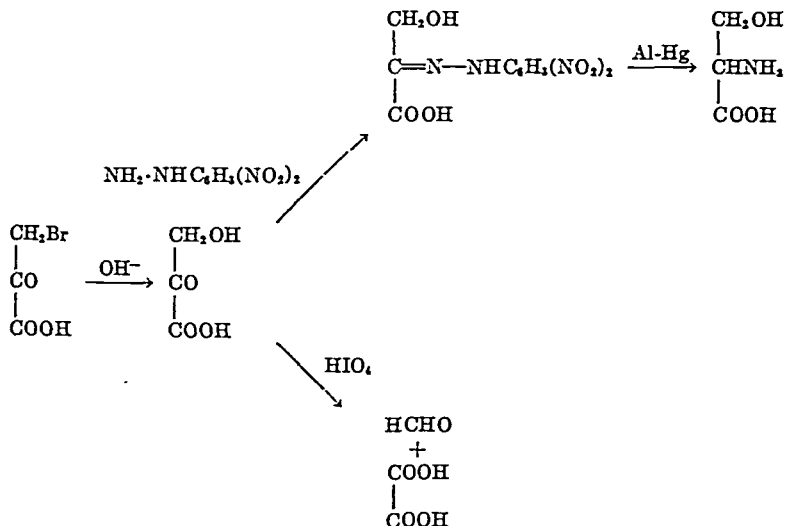


alcohol, afforded the 2,4-dinitrophenylhydrazone of ethoxypyruvic acid (V).

Freshly prepared solutions of hydroxypyruvic acid are rapidly decomposed by periodic acid with the consumption of 1 mole of oxidizing agent and the production of about 0.9 mole each of formaldehyde and of oxalic

acid per mole of bromopyruvic acid used as the starting material. Taken together with the reduction of hydroxypyruvic acid 2,4-dinitrophenylhydrazone to *dl*-serine, this proves conclusively that 90 per cent of the hydrolysis product of bromopyruvic acid is indeed hydroxypyruvic acid (II). The reactions leading from bromopyruvic acid to serine on the one

Scheme 1



hand and to formaldehyde and oxalic acid on the other are summarized in Scheme 1.

#### *Behavior of Hydroxypyruvic Acid toward Strong Alkali (Table I)*

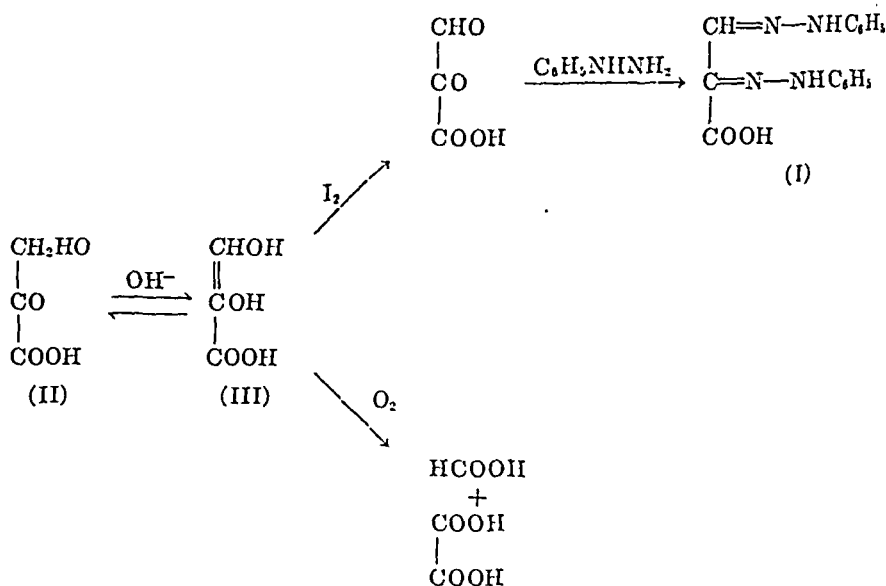
When hydroxypyruvic acid solutions in a nitrogen atmosphere are treated with sodium hydroxide (0.02 to 0.8 *N*) and are acidified after  $\frac{1}{2}$  hour, they show an ability to consume iodine (15) corresponding to the formation of appreciable amounts (10 to 64 per cent) of enediol. This solution reduces Benedict's reagent at room temperature. That this strong reducing agent is dihydroxyacrylic acid (III) is shown by its behavior. After oxidation with iodine, treatment of the solution with phenylhydrazine acetate at room temperature affords an almost quantitative yield, based on the enediol content, of the osazone of mesoxalic acid semialdehyde (I), mentioned previously. Moreover, when a solution of hydroxypyruvic acid in 0.8 *N* sodium hydroxide is shaken with oxygen, about 0.7 mole of oxygen is consumed and equivalent amounts of oxalic and formic acids are formed for each mole of starting material. The reactions involved are presented in Scheme 2.

TABLE I

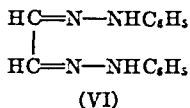
*Formation of Enediol from Hydroxypyruvic Acid*

In each experiment the calculated amount of 9.16 N sodium hydroxide was added to 0.01 mole of sodium hydroxy pyruvate in 200 cc. of water, in order to obtain the indicated alkali concentration. The solutions were kept for 30 minutes in a nitrogen atmosphere before removal of aliquots, which were then acidified and titrated with 0.1 N iodine solution.

Experiment No.	Normality of solution with respect to NaOH	Iodine consumption per 0.01 mole hydroxypyruvic acid	Enediol formed
		<i>m.eq.</i>	<i>per cent</i>
1	0.01	0.72	3.6
2	0.02	2.0	10
3	0.09	7.0	35
4	0.22	9.2	46
5	0.83	12.8	64
6	1.5	12.5	63

*Scheme 2*

When phenylhydrazine acetate at room temperature is added to the acidified solution before oxidation with iodine, amorphous bulky precipitates result from which pure glyoxalosazone (VI) can be secured in yields roughly dependent on the amount of enediol formed. This derivative probably arises as a result of the decarboxylation of compound III. Compound IV, possibly also present in the alkaline solutions of hydroxypyruvate, is known to give glyoxalosazone with phenylhydrazine at 60° (14). The



course of the reaction between enediols and phenylhydrazine is not entirely clear. Ascorbic acid yields the osazone of dehydroascorbic acid (16, 17), while reductone (dihydroxyacrylic aldehyde) does not yield a crystalline derivative with phenylhydrazine unless it is first oxidized with iodine to dehydroreductone (18).

#### *Behavior of Hydroxypyruvic Acid toward Weak Alkali*

The complex transformations of this acid induced by 0.01 *N* alkali were studied in detail. The difficulties experienced in the interpretation of the results were not unexpected in view of the reactivity of this hydroxyketo acid and the multiplicity of reaction products to which it could give rise.

Hydroxypyruvic acid consumes 1 mole of periodic acid and yields 1 mole each of formaldehyde and oxalic acid; after treatment with 0.01 *N* sodium hydroxide under nitrogen for 2 to 4 hours it rapidly reduces 1.5 to 1.7 moles of periodate, but then yields only 0.4 mole of oxalic acid and less than 0.1 mole of formaldehyde. At the same time carbon dioxide is produced, irrespective of the duration of contact with alkali, in an amount corresponding to about 0.4 mole (Table II). Without previous contact with alkali hydroxypyruvic acid yields practically no carbon dioxide on treatment with periodate. (Compare Experiment 1 in Table II and the discussion in the following paper (19).) The disappearance of formaldehyde indicates the abolition of a primary alcohol group; the simultaneous production of roughly 0.5 mole of oxalic acid reveals the presence of a grouping such as  $-\text{CHOH}\cdot\text{CO}\cdot\text{COOH}$ . The production of carbon dioxide by periodate points, in the light of the discussion presented in the following communication (19), to the formation of a polyhydroxy acid  $-(\text{CHOH})_2\cdot\text{COOH}$ .

The various findings outlined here suggest the occurrence of an aldol condensation between 2 or more molecules of II, or possibly between II and IV, analogous to the condensation of glyceraldehyde and dihydroxyacetone to *dl*-fructose and *dl*-sorbose (20, 21) or of *d*-glyceraldehyde to *d*-fructose and *d*-sorbose (22). The experimental results could be explained by the formation of a compound of the type of an  $\alpha$ -ketotrihydroxyadipic acid.<sup>1</sup>

<sup>1</sup> The reactivity of hydroxypyruvic acid may prove of biological interest. In animals known to synthesize ascorbic acid (*e.g.* the rat), it is possible that the pathway of the reaction is via an asymmetric aldol condensation between *l*-glyceraldehyde and hydroxypyruvic acid to 2-keto-*l*-gulonic acid, followed by enolization and lactonization to the vitamin.

The prolonged contact of hydroxypyruvic acid with 0.01 *N* alkali causes a slow spontaneous decarboxylation accompanied by a drop in pH (Table II). After 72 hours 1 mole of II gives rise to almost 0.5 mole of carbon dioxide. This formation of carbon dioxide may be due to a gradual decarboxylation of a  $\beta$ -keto acid, which is in tautomeric equilibrium with the  $\alpha$ -keto acid originally produced by weak alkali:  $-\text{CHOH}\cdot\text{CO}\cdot\text{COOH} \rightleftharpoons -\text{COH}=\text{COH}\cdot\text{COOH} \rightleftharpoons -\text{CO}\cdot\text{CHOH}\cdot\text{COOH} \rightarrow -\text{CO}\cdot\text{CH}_2\text{OH} + \text{CO}_2$ . A simple tautomeric shift of II to the  $\beta$ -aldehyde acid (IV) appears

TABLE II  
*Action of 0.01 N Alkali on Hydroxypyruvic Acid*

Experiment No.*	Time after alkalization	pH	Moles of carbon dioxide per mole of hydroxypyruvate produced by spontaneous decarboxylation	Products of oxidation with $\text{NaIO}_4$ , mole per mole of hydroxypyruvate		
				Carbon dioxide	Formaldehyde (as dimedon derivative)	Oxalic acid
	<i>hrs.</i>					
1		7.0	0.050	0.094	0.87	0.85
2a	0	11.6				
2b	4	11.1	0.066	0.37	0.07	0.41
2c	24	9.9	0.27	0.39	0.14	0.26
2d	48	9.4	0.40	0.40	0.22	0.19
2e	72	9.2	0.47	0.36	0.25	0.15
2f	96	9.0	0.50	0.38	0.25	0.15
2g	120				0.26	0.15
2h	144	9.0	0.53	0.34		

\* The freshly prepared 0.05 *M* sodium hydroxypyruvate solution (Experiment 1) was made 0.01 *N* with respect to alkali (Experiment 2a). The progressive decrease in pH, increase in carbonate, and the products of oxidation with sodium periodate (carbon dioxide, formaldehyde, and oxalic acid) are recorded as Experiments 2b to 2h. The values of carbonate reported in the fourth column were subtracted from the total produced in the periodate oxidation to give the figures reported in the fifth column. Corrections are made for the values of carbon dioxide found in Experiment 1.

unlikely, since under those circumstances 1 rather than 0.5 mole of carbon dioxide should be produced. The view that the spontaneous decarboxylation of the condensation product of hydroxypyruvic acid leads to the formation of the grouping  $-\text{CO}\cdot\text{CH}_2\text{OH}$ , *i.e.* of a primary alcohol group, is borne out to a large extent by the trend in the amounts of formaldehyde and oxalic acid produced by the action of periodate on the condensation product at different stages of spontaneous decarboxylation (Fig. 1). In measure with the increase in spontaneously produced carbon dioxide, the formaldehyde values rise from 0.07 to 0.26 mole and the yields in oxalic acid drop from 0.4 to 0.15 mole (Table II). Attempts, briefly summarized in the experimental part, to determine in a more definite



manner the type of carbon skeleton produced by the condensation of II have so far not led to conclusive results.<sup>2</sup>

*Synthesis of  $\beta$ -Hydroxy- $\alpha$ -ketobutyric Acid*—The keto acid corresponding to *dl*-threonine, *dl*- $\beta$ -hydroxy- $\alpha$ -ketobutyric acid,  $\text{CH}_3\cdot\text{CHOH}\cdot\text{CO}\cdot\text{COOH}$ , was also synthesized.  $\beta$ -Bromo- $\alpha$ -ketobutyric acid, prepared from  $\alpha$ -ketobutyric acid, yielded, by careful hydrolysis, solutions of the desired sub-

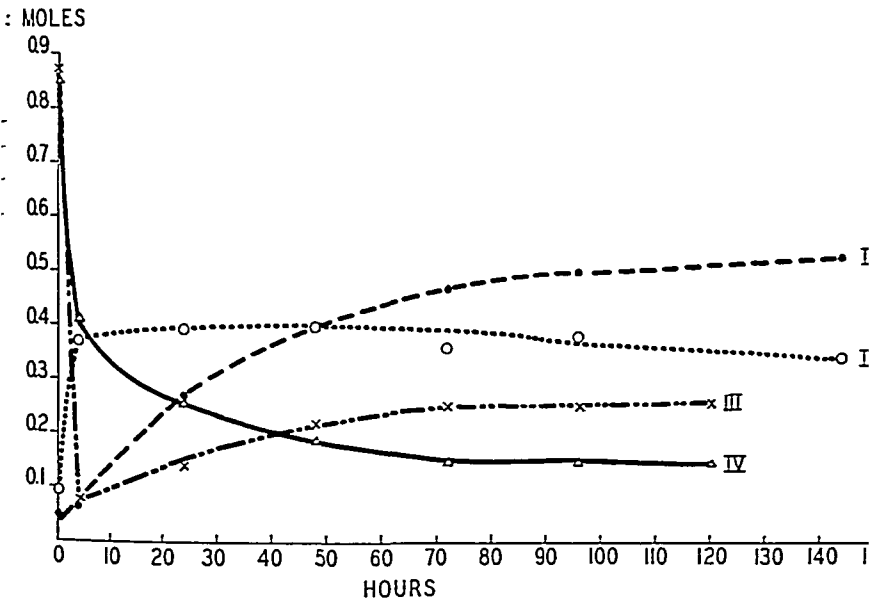


FIG. 1. Behavior of condensation product produced from hydroxypyruvic acid by 0.01 *N* alkali. Curve I, carbon dioxide formed by spontaneous decarboxylation. Curves II, III, and IV represent the products of periodate oxidation: Curve II, carbon dioxide; Curve III, formaldehyde; Curve IV, oxalic acid. Ordinate, moles of reaction products per mole of hydroxypyruvic acid; abscissa, time elapsed after addition of alkali.

stance. The 2,4-dinitrophenylhydrazone was obtained in good yield.<sup>3</sup> Oxidation of the solution of this hydroxyketo acid by periodate produced

<sup>2</sup> It is evident from the extreme lability of II to alkali that it cannot have arisen directly from the alkaline decomposition products of cellulose nitrate mentioned in the beginning of this paper. This agrees with the conclusions reached by Kenyon in a careful investigation of the reactions involved in the degradation of cellulose nitrate by alkali (23); compare (24).

<sup>3</sup> The synthesis of a compound described as  $\beta$ -hydroxy- $\alpha$ -ketobutyric acid was reported recently (25). There are, however, several points of difference between this product and the compound described here.

oxalic acid and acetaldehyde almost quantitatively, with the consumption of 1 mole of oxidant.

# EXPERIMENTAL

## Bromopyruvic Acid

*Preparation*—This compound was prepared according to a modification of the procedure described by Ward (26). 1 mole (88.1 gm.) of pyruvic acid (m.p. 13.6–13.8°) was heated to 50° in a 3-neck flask equipped with ground glass joints, and 160 gm. (1 mole) of bromine, previously dried by shaking with concentrated  $\text{H}_2\text{SO}_4$ , were added dropwise with stirring and exclusion of moisture. The heat of the reaction was usually sufficient to keep the temperature at 50°; external temperature control was resorted to when necessary. The thick fuming sirup was immediately poured into a large crystallizing dish, the flask washed with a little hot benzene, and the washing added to the main product. Sometimes the material set in the flask to a fuming crystal mass which was dissolved in a small amount of hot benzene. The mixture was placed in a vacuum desiccator over moist NaOH pellets, and the solvent removed by suction. On the next day the material was ground to a fine powder<sup>4</sup> and kept *in vacuo* for 48 to 72 hours, with frequent renewal of the alkali, until no more fumes of HBr were given off. The yield was 164 gm. (98 per cent) of white crystals melting at 70°. Crystallization from dry chloroform (1 cc. per gm.) with the aid of mechanical stirring to prevent caking yielded 135 to 140 gm. of hexagonal prisms melting at 74°.<sup>5</sup>

$\text{C}_3\text{H}_3\text{O}_3\text{Br}$ .	Calculated.	C 21.6, H 1.8, Br 47.9
167.0	Found.	" 21.4, " 1.6, " 47.8

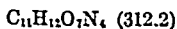
*Bromopyruvic Acid 2,4-Dinitrophenylhydrazone*—5 gm. (0.03 mole) of bromopyruvic acid dissolved in 100 cc. of water were treated with a solution of 5.9 gm. (0.03 mole) of 2,4-dinitrophenylhydrazine in 400 cc. of 2 N HBr with vigorous stirring and cooling in ice water. After 1 hour the precipitate was filtered off, washed with cold 2 N HBr and cold water, and dried *in vacuo* over  $\text{P}_2\text{O}_5$ . The product weighed 9.8 gm. (95 per cent) and melted at 180°. It was analytically pure. Crystallization from dioxane gave fine yellow needles of unchanged melting point.

$\text{C}_7\text{H}_7\text{O}_6\text{N}_4\text{Br}$ .	Calculated.	C 31.2, H 2.0, N 16.1, Br 23.0
347.1	Found.	" 31.2, " 2.1, " (Dumas) 16.1, Br. 23.2

<sup>4</sup> The unstable intermediate compound (26) as well as the HBr-free bromopyruvic acid is a strong vesicant.

<sup>5</sup> The melting points, reported without correction, were determined with the Fisher-Johns apparatus. It is noteworthy that bromopyruvic acid, when observed under these conditions, forms a transparent glass at about 60° (which may account for the reported melting point of 59° (26)) and melts to an oil at 74°.

*Ethoxypyruvic Acid 2,4-Dinitrophenylhydrazone*—1 gm. (2.9 mm) of the bromopyruvic acid hydrazone was dissolved in 100 cc. of 80 per cent alcohol and titrated electrometrically with 0.2 N aqueous NaOH. When a pH of 8 was reached, 2 equivalents of alkali had been consumed and the titration was stopped. Acidification with 2 N HCl gave 0.82 gm. (91 per cent) of the *ethoxypyruvic acid 2,4-dinitrophenylhydrazone*, melting at 153–155° (the solidified melt had a melting point of 163–166°). Crystallization from ethyl acetate yielded yellow needles melting at 154° (second m.p. 164–167°). The mixture of this compound with *hydroxypyruvic acid 2,4-dinitrophenylhydrazone* (see below) melted at 138–140°. A solution of the hydrazone in 80 per cent ethanol was titrated electrometrically with 0.1 N alkali.



Calculated. C 42.3, H 3.9, N 18.0 —OC<sub>2</sub>H<sub>5</sub>, 14.4, neutralization equivalent 312

Found. " 42.1, " 3.9, " (Dumas) 18.0, —OC<sub>2</sub>H<sub>5</sub>, 13.4, neutralization equivalent 305

### *Hydroxypyruvic Acid*

*Preparation*—The following procedure, out of several tried (e.g. treatment with silver carbonate or with potassium acetate in acetic acid-acetic anhydride), proved the most successful. A solution of 4.175 gm. (0.025 mole) of bromopyruvic acid in 30 cc. of water was placed in a 500 cc. volumetric flask and 465 cc. of 0.107 N NaOH or KOH (2 equivalents) were added in such a way as to keep the pH below 8.5. The first 400 cc. could be added slowly in 1 portion with shaking to give a pH of 6.8. The remainder was added in small volumes at intervals, additions being made whenever the pH fell from 8.5 to 7. These solutions, 0.05 M with respect to hydroxypyruvic acid after adjustment to volume, were used in all experiments.

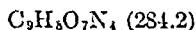
Solutions of hydroxypyruvate (II) reduced Benedict's solution on gentle warming and silver nitrate at room temperature. Bromide could be removed by adding a solution of the required amount of AgNO<sub>3</sub>, although not without some reduction. The filtrate gave an insoluble, amorphous lead salt. The acid could not be extracted from its aqueous solution by ether, although it is soluble in it as shown below. No significant reaction with ferric chloride was observed, unless alkali was added, when a deep violet solution resulted.

The addition of phenylhydrazine acetate to solutions of II gave, at room temperature, brown amorphous precipitates which were ill defined and bore little resemblance to the well characterized osazone I. Attempts to prepare an osazone at a higher temperature led to deeply colored products.

Crude preparations of the free acid could be made by adding 1 equivalent of 10 N HBr to the concentrated aqueous solution of potassium hydroxy-

pyruvate and evaporating to dryness *in vacuo* in a current of  $N_2$  at room temperature. Ether was added to the residue and the insoluble salts were repeatedly washed with ether, when a colorless ethereal solution was obtained. The evaporation residue of the ether solution formed a straw-colored sirup which, dissolved immediately in water, yielded solutions which showed a 90 per cent recovery by acidimetric titration and 80 per cent by periodate consumption.

*Hydroxypyruvic Acid 2,4-Dinitrophenylhydrazone*—5 gm. (0.03 mole) of bromopyruvic acid, were hydrolyzed with 600 cc. of 0.1  $N$  KOH, as described above, and 5.5 gm. (0.028 mole) of 2,4-dinitrophenylhydrazine in 500 cc. of 2  $N$  HCl were added. After being chilled overnight the semi-crystalline precipitate was filtered off, washed with 2  $N$  HCl and water, and dried *in vacuo* over  $P_2O_5$ . The material, which was free of halogen, weighed 7.5 gm. (95 per cent on the basis of the 2,4-dinitrophenylhydrazine added) and melted at 158–160°. Crystallization from ethyl acetate, followed by the thorough removal of the solvent at 0.1 mm. of Hg and 80°, gave 4.4 gm. of orange needles, melting at 162°. An additional amount of equally pure substance could be obtained from the mother liquor by crystallization from 3 volumes of ethyl acetate and 1 volume of ligroin (b.p. 60–90°). A solution of the hydrazone in 80 per cent alcohol was titrated electrometrically with 0.01  $N$  alkali.



Calculated. C 38.0, H 2.8, N 19.7, neutralization equivalent 284

Found. " 38.0, " 2.8, " (Dumas) 19.8, neutralization equivalent 282

*Reduction of Hydroxypyruvic Acid 2,4-Dinitrophenylhydrazone to dl-Serine*—The procedure used was patterned after the method for the reduction of phenylhydrazones (27). To 3 gm. (0.0105 mole) of the hydrazone dissolved in 100 cc. of 80 per cent ethanol was added aluminum amalgam (28) prepared from 10 gm. of aluminum turnings. The reaction mixture became warm and was cooled in ice water with occasional shaking for 30 minutes, followed by shaking at room temperature for 16 hours. The precipitate was centrifuged off and extracted three times with boiling water; the solution was evaporated *in vacuo* to dryness and the residue extracted with hot water. The deep brown color of the combined aqueous extracts (250 cc.) was removed by continuous extraction with ether for 3 days. The water layer was treated with norit and the filtrate evaporated *in vacuo* to a very small volume. Addition of absolute alcohol produced 300 mg. (27 per cent) of slightly colored crystals decomposing at 240°. When a small sample was heated with *p*-nitrobenzoyl chloride in pyridine, followed by the addition of 10 per cent  $Na_2CO_3$ , the strong pink color characteristic for serine in this general test for  $\alpha$ -amino acids was observed (29). The crude material, which was almost analytically pure, was dissolved in 20 cc. of water and treated with charcoal (Darco G-60) in the

cold. The filtrate was concentrated *in vacuo* to a very small volume and an excess of absolute alcohol added when *dl*-serine was obtained in the form of white crystals weighing 260 mg.

$C_3H_7O_2N$  (105.1)

Calculated. C 34.3, H 6.7, N 13.3

Found. " 33.9, " 6.9, " (Dumas) 13.3, amino N 13.3, amino acid N (30) 13.3

*3-Phenyl-5-hydroxymethylhydantoin*—The attempt to effect an additional characterization of the *dl*-serine by conversion to  $\alpha$ -hydroxymethyl- $\delta$ -phenylhydantoic acid (31) led in our hands directly to the corresponding hydantoin (32). The same observation was made with an authentic specimen of *dl*-serine. Analyses of both hydantoin preparations (respectively designated Hydantoins 1 and 2) are reported below. To 100 mg. of *dl*-serine in 0.5 cc. of water 1 cc. of *N* NaOH and 0.10 cc. of phenyl isocyanate (in two portions) were added with cooling in an ice-salt mixture. The mixture was kept at 0° for 1 hour, acidified with concentrated HCl, cleared by centrifugation, and concentrated to a small volume in an evacuated desiccator over  $P_2O_5$ . The precipitate was recrystallized from a small volume of water when crystals melting at 162–163° (Hydantoin 1) and 166–167° (Hydantoin 2) were obtained. A mixture of both specimens melted at 163–166°.

$C_{10}H_{10}O_2N_2$ .	Calculated.	C 58.2, H 4.9, N 13.6
206.2	Found, Hydantoin 1.	" 58.0, " 5.0, " (Dumas) 13.3
	" "	2. " 58.0, " 4.9, " ( " ) 13.5

*Oxidation with Periodic Acid*—5 cc. of a freshly prepared 0.05 *M* solution of sodium hydroxypyruvate were treated either with periodate, according to procedures (a) and (b), as described in the following paper (19), or under strongly acid conditions<sup>6</sup> with 1 cc. of 0.5 *M* periodic acid in the presence of 10 cc. of water. In the last mentioned procedure the unused reagent was determined according to the method of Malaprade (33). In all experiments the consumption of periodate was 0.95 to 1.0 mole per mole of hydroxypyruvic acid, when acted upon by the reagent for 10 to 150 minutes. The oxidation products were 0.9 mole of formaldehyde (as the dimedon derivative melting at 189–191°) and 0.86 mole of oxalic acid. (For the isolation procedures, compare (19).)

#### *dl*- $\beta$ -Hydroxy- $\alpha$ -ketobutyric Acid

*dl*- $\beta$ -Bromo- $\alpha$ -ketobutyric Acid— $\alpha$ -Ketobutyric acid, which served as starting material, was prepared by hydrolysis (34) of ethyl ethoxalyl-

<sup>6</sup> Since the 0.05 *M* hydroxypyruvate solutions were also 0.05 *M* with respect to bromide, small amounts of bromine were liberated by the periodic acid in the strongly acid solutions. These amounts were not sufficient, however, to influence the results.

propionate (35). Bromination of 51 gm. (0.5 mole) of keto acid, as described above, gave 87 gm. (96 per cent) of crude material. By crystallization from a mixture of 40 cc. of chloroform and 120 cc. of ligroin (b.p. 60–90°), 71 gm. of strongly hygroscopic white plates were obtained, which melted at 60° (determined in a closed tube).

$C_4H_5O_3Br$ .	Calculated.	C 26.6, H 2.8, Br 44.1
181.0	Found.	" 26.8, " 2.8, " 43.7

*dl*- $\beta$ -Hydroxy- $\alpha$ -ketobutyric Acid—Solutions of this acid were prepared by the careful addition (with electrometric control) of 200 cc. of 0.1 *N* alkali (2 equivalents) to a solution of 1.81 gm. (0.01 mole) of *dl*- $\beta$ -bromo- $\alpha$ -ketobutyric acid in 20 cc. of water in such a manner as to maintain the pH between 7 and 8.5.

*dl*- $\beta$ -Hydroxy- $\alpha$ -ketobutyric Acid 2,4-Dinitrophenylhydrazone—A solution of the acid (0.01 mole in a volume of 220 cc.), freshly prepared as described in the preceding paragraph, was cooled to 5° and a cold solution of 2.0 gm. (0.01 mole) of 2,4-dinitrophenylhydrazine in 300 cc. of 2 *N* HCl was added. The mixture was cooled overnight and the yellow precipitate washed with cold 2 *N* HCl and cold water. It weighed 2.6 gm. (87 per cent yield). Crystallization from 50 cc. of ethyl acetate gave 1.3 gm. of yellow needles, melting at 157–158°.

$C_{10}H_{10}O_7N_4$  (298.2)

Calculated. C 40.3, H 3.4, N 18.8, neutralization equivalent 298

Found. " 40.4, " 3.4, " (Dumas) 18.7, neutralization equivalent 297

*Oxidation with Periodic Acid*—The oxidation of 5 cc. of a 0.05 *M* hydroxyketobutyric acid solution in the presence of 15 cc. of *M* sodium bicarbonate, with 1 cc. of 0.43 *M* sodium periodate, resulted in the consumption of 0.25 mm of the oxidant in 30 minutes. A similar oxidation of 1.25 mm of the keto acid yielded, by the isolation procedure described in the following paper (19), 0.181 gm. of calcium oxalate monohydrate (1.24 mm), which required 23.8 cc. of 0.1 *N* potassium permanganate for oxidation.

For the isolation of the dimedon derivative of acetaldehyde, 25 cc. of the keto acid solution (1.25 mm) were oxidized with 4 cc. of 0.43 *M* sodium periodate, in the presence of 15 cc. of *M* sodium bicarbonate, in a tightly stoppered flask. Half an hour after the addition of 1 cc. of 2 *M* sodium arsenite, in order to destroy unused oxidant, the solution was made acid to methyl red with glacial acetic acid and 200 cc. of a 0.4 per cent dimedon solution were added. The acetaldehyde dimedon derivative weighed 0.345 gm. and melted at 139°. The yield corresponded, after correction for solubility of the dimedon derivative, to 96 per cent of the expected amount.

### *Action of Strong Alkali on Hydroxypyruvic Acid*

**Formation of Enediol**—To 200 cc. of 0.05  $M$  hydroxypyruvate varying amounts of 9.16  $N$  NaOH were added with shaking in a nitrogen atmosphere.<sup>7</sup> After 30 minutes 5 cc. samples were withdrawn, cooled, acidified with 2  $N$  HCl, and titrated with a 0.1  $N$  iodine solution. The formation of dihydroxyacrylic acid (III), progressing with the strength of the alkali until an enediol concentration of about 64 per cent is reached, is presented in Table I. The same results were obtained with solutions permitted to remain alkaline for 2 hours.

**Isolation of Glyoxalosazone**—Portions (180 cc.) of the alkaline solutions, represented as Experiments 2 to 5 in Table I, were acidified with glacial acetic acid and treated with 3 cc. of phenylhydrazine in 50 per cent acetic acid at room temperature. Bulky brown precipitates were obtained in a few minutes and were filtered off after 1 hour. The crude dry products weighing 0.4, 0.9, 0.96, and 1.0 gm., respectively, were taken up in 25 cc. of absolute ethanol, decolorized with charcoal (Darco G-60), and crystallized by the addition to the filtrate of sufficient water to produce a slight turbidity. The yields of the light yellow rectangular plates of pure *glyoxalosazone* melting at 169° were 0.14, 0.30, 0.30, and 0.37 gm., respectively.

$C_{11}H_{11}N_4$ .	Calculated.	C 70.6, H 5.9, N 23.5
238.3	Found.	" 70.4, " 5.8, " (Dumas) 23.5

**Oxidation of Enediol with Iodine; Isolation of Osazone of Mesoxalic Acid Semialdehyde**—A 0.05  $M$  solution of hydroxypyruvic acid was made 0.8  $N$  with respect to NaOH, as described above (Experiment 5, Table I). After the oxidation with iodine, a portion of the solution, corresponding to 0.75  $mM$  of enediol, was treated with 3 cc. of phenylhydrazine in 15 cc. of 50 per cent acetic acid. After 2 hours at room temperature the precipitate was filtered off and dried; it weighed 200 mg. and melted at 214–215°. Crystallization from chloroform gave 110 mg. of the orange *osazone of mesoxalic acid semialdehyde (I)* melting with decomposition at 222–223° (36). A mixture with a specimen obtained by the alkaline decomposition of cellulose nitrate (2) melted at 221–222°.

$C_{13}H_{11}O_2N_4$ .	Calculated.	C 63.8, H 5.0, N 19.9
282.3	Found.	" 63.6, " 5.1, " (Dumas) 19.9

**Action of Oxygen on Enediol**—200 cc. of a 0.05  $M$  solution of sodium hydroxypyruvate (10  $mM$ ) were placed in a hydrogenation flask, cooled in an ice bath, and made 0.85  $N$  with respect to alkali by the addition, in an atmosphere of nitrogen, of 20 cc. of 9.4  $N$  sodium hydroxide (which, as shown in Table I, brought about the formation of about 65 per cent of

<sup>7</sup> The alkaline solutions became golden yellow after a few minutes. Acidification discharged the color.

enediol). After 30 minutes the flask was connected to a large gas burette filled with oxygen, the nitrogen was displaced by oxygen, and the flask was shaken for 45 minutes. The consumption of oxygen ceased after 10 minutes when about 7 mm of oxygen had been taken up and the originally yellow color of the solution had disappeared. The remaining solution, which was now inert to periodic acid, contained 16 milliequivalents of newly formed acids.

The contents of the hydrogenation flask were made up to 250 cc. and 25 cc. aliquots (corresponding to 1 mm of hydroxypyruvate) were acidified to methyl red with acetic acid. Oxalic acid was precipitated as the calcium salt, of which 90 mg. (0.70 mm) were obtained, requiring 12.9 cc. of 0.1 N  $\text{KMnO}_4$  (calculated 14.0 cc.). A 100 cc. aliquot was brought to pH 1.5 with sulfuric acid and extracted continuously for 40 hours with ether, the solvent in the boiling flask being placed over a few cc. of 10 per cent sodium carbonate. The alkaline layer was analyzed for formic acid by means of mercuric chloride (37, 38); it contained an amount corresponding to 0.62 mole of formic acid per mole of hydroxypyruvate.

#### *Action of Weak Alkali (0.01 N) on Hydroxypyruvic Acid*

The solutions were prepared by adding 1 cc. of 10 N NaOH to 800 cc. of a 0.05 M hydroxypyruvate solution in a nitrogen atmosphere. The fresh solutions had a pH of 11.6.

The consumption of periodate (determined in 5 cc. aliquots) and production of oxalic acid and formaldehyde (determined in 20 cc. aliquots) were followed by the methods described in the following paper (19).

Oxidative decarboxylation (19) was estimated from the amount of carbon dioxide produced when 1 cc. of solution, 1 cc. of water, and 1 cc. of 0.43 M sodium periodate were allowed to remain for 1 hour at room temperature in the Van Slyke-Neill manometric apparatus. Spontaneous decarboxylation was determined in the same manner with the omission of oxidant.

When the 0.05 M hydroxypyruvate solutions were exposed at room temperature to 0.01 N alkali for 2 to 4 hours,<sup>8</sup> the consumption of periodate during 60 minutes rose to 1.5 to 1.7 moles per mole of keto acid initially present and remained at about 1.5 moles throughout the period of the experiment recorded in Table II, which summarizes the data on the course of the transformation of hydroxypyruvic acid by weak alkali.

The alkalization of solutions of hydroxypyruvic acid with  $\text{Ba}(\text{OH})_2$ , the direct hydrolysis of bromopyruvic acid with an excess of baryta (pH

<sup>8</sup> In contrast to the experiments carried out with strong alkali, no glyoxalosazone or any other definite derivative could be isolated upon the treatment of hydroxypyruvate solutions, exposed to 0.01 N alkali, with phenylhydrazine or 2,4-dinitrophenylhydrazine. Enediol formation was minimal under these conditions. (Compare Experiment 1 in Table I.)



11), or the addition of barium salts to weakly alkaline solutions of hydroxypyruvic acid yielded an insoluble amorphous barium salt containing Ba 45.7, C 18.7, H 1.3 (corrected for a water content of 9.4 per cent in the air-dried material).

A molar solution of hydroxypyruvic acid (50 cc.), prepared by concentration as described before, was held at pH 11 for 14 hours at room temperature. 1 M equivalent of sodium chlorite was then added (39) and the solution maintained for 7 days at pH 5 to 7 by the addition of acetic acid. The white precipitate produced by the addition of calcium chloride to the solution (freed of  $\text{ClO}_2$  by evacuation at room temperature) weighed 1.5 gm. and contained Ca 18.9, C 26.6, H 2.3 (corrected for a water content of 13.2 per cent in the air-dried sample). The calcium salt reduced hot Benedict's solution. This salt consumed, after the removal of calcium, 3 moles of sodium periodate and produced 0.8 mole of oxalic acid and 1.2 moles of carbon dioxide per atom of Ca. The oxidation and other analytical data are in fair agreement with those for the calcium salt of a 2-keto-3,4-dihydroxyglutaric acid (calculated, Ca 18.6, C 27.8, H 1.9).

Our indebtedness to Mr. W. Saschek for the microanalyses is gratefully acknowledged.

#### SUMMARY

The synthesis of  $\beta$ -hydroxy- $\alpha$ -keto acids was achieved by the careful hydrolysis of the corresponding  $\beta$ -bromo- $\alpha$ -keto acids. Bromopyruvic and *dl*- $\beta$ -bromo- $\alpha$ -ketobutyric acids yielded solutions of hydroxypyruvic and *dl*- $\beta$ -hydroxy- $\alpha$ -ketobutyric acids respectively. The 2,4-dinitrophenylhydrazone of bromopyruvic acid could be converted into the corresponding derivative of ethoxypyruvic acid.

Both hydroxyketo acids gave crystalline 2,4-dinitrophenylhydrazones. The structure of synthetic hydroxypyruvic acid was proved through its cleavage by periodic acid to formaldehyde and oxalic acid and by the reduction of its dinitrophenylhydrazone to *dl*-serine. Similarly, periodic acid produced acetaldehyde and oxalic acid from hydroxyketobutyric acid.

In the presence of strong alkali (0.8 N) hydroxypyruvic acid quickly formed the enediol, dihydroxyacrylic acid, which could be made to yield glyoxalosazone or, after oxidation with iodine, the phenylosazone of mes-oxalic acid semialdehyde.

The exposure of hydroxypyruvic acid to weak alkali (0.01 N) produced a rapid and complex series of tautomeric changes and condensations. The nature of the reaction product is discussed in detail, and the evidence for its formation by an aldol condensation, perhaps analogous to that undergone by glyceraldehyde under similar conditions, is presented. Some im-

plications with respect to the biological significance of the reactivity of hydroxypyruvic acid are indicated.

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# ON OXIDATIVE DECARBOXYLATIONS WITH PERIODIC ACID\*

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The behavior of hydroxypyruvic acid,  $\text{CH}_2\text{OH}\cdot\text{CO}\cdot\text{COOH}$ , and some of its transformation products toward periodic acid, treated in detail in the preceding paper (1), suggested an orienting study of the action of this oxidant on glyoxylic acid,  $\text{CHO}\cdot\text{COOH}$ , and other  $\alpha$ -keto acids or on compounds giving rise to such keto acids, following oxidation by periodate.

While  $\alpha$ -glycols,  $\alpha$ -ketols,  $\alpha$ -diketones, and  $\alpha$ -keto aldehydes are oxidized by periodic acid,  $\alpha$ -keto acids are assumed to be inert to this reagent under the conditions commonly applied. (Compare the survey of the literature in (2).) Tartaric acid is reported to consume only 1 mole of periodic acid (3, 4); the further oxidation of the resulting 2 moles of glyoxylic acid to formic acid and carbon dioxide is claimed to require 36 to 48 hours for completion at room temperature (5). The very slow development of carbon dioxide (within 1 to 4 days) in the course of the oxidation of keto sugars by periodic acid has been attributed to the gradual breakdown of the glyoxylic acid arising as a primary product of the oxidation (6). Similarly, glyoxylic acid originating from the action of periodate on  $\beta$ -hydroxy- $\alpha$ -amino acids has been reported to be slowly attacked by the oxidant (7), though this action was shown to proceed at a faster rate in the presence of weak alkali (8). Pyruvic acid likewise is degraded slowly (9).

The following discussion will attempt to illustrate the extent to which the oxidative decarboxylation by periodate of  $\alpha$ -keto acids and compounds giving rise to them may be useful as a diagnostic method. Tartaric acid, as the most thoroughly investigated example of a polyhydroxy acid, will be taken up first, followed in turn by various  $\alpha$ -keto acids, hydroxyamino acids, polyhydroxy acids, keto sugars, polycarbonyl compounds, and malonic and tartronic acids.

## *Oxidation of d-Tartaric Acid*

*Periodate Consumption*—A closer study of the oxidation of tartaric acid by periodate showed it to be dependent upon the concentration of the oxi-

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† This report is from a dissertation submitted by David B. Sprinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

dant, the pH of the reaction mixture, and the temperature. The results are summarized in Table I. Since the primary oxidation of tartaric acid as an  $\alpha$ -glycol is very rapid (5), these experiments furnish an indication of the rates at which glyoxylic acid is oxidized under various conditions.

TABLE I  
*Consumption of Periodate by Tartaric Acid*

Experiment No.*	Diluent	Oxidant	Initial molar ratio of oxidant to tartaric acid	Duration of oxidation	Oxidant consumed per mole of tartaric acid
				<i>min.</i>	<i>moles</i>
1	M NaHCO <sub>3</sub>	HIO <sub>4</sub>	2.2	25	1.9
2	" "	"	2.2	45	2.0
3	" "	"	4.3	20	2.8
4	" "	"	4.3	30	2.9
5	" "	"	4.3	60	3.0
6†	" " "	"	2.2	10	1.0
7†	" "	"	2.2	20	1.1
8	0.13 N H <sub>2</sub> SO <sub>4</sub>	"	2.2	5	0.68
9	0.13 " "	"	2.2	10	0.84
10	H <sub>2</sub> O	"	2.2	5	1.1
11	"	"	2.2	15	1.1
12	"	"	2.2	30	1.3
13	"	"	4.3	120	2.2
14	"	NaIO <sub>4</sub>	1.7	30	1.3
15	"	"	3.6	30	2.2
16	"	"	3.6	60	2.6
17	"	"	3.6	90	2.7

\* 1 mole of tartaric acid requires 1 mole of periodate for the production, and additional 2 moles for the oxidation, of glyoxylic acid. In each experiment 5 cc. of 0.05 M solution of tartaric acid, 15 cc. of the diluent indicated, and 1 or 2 cc. of 0.54 M HIO<sub>4</sub> or of 0.43 M NaIO<sub>4</sub> were used. The oxidation was, with the exception of Experiments 6 and 7, carried out at room temperature. In Experiments 1 to 7 the excess periodate was determined by the method of Fleury and Lange (10). In Experiments 8 to 17 the excess periodate was determined in acid solution, according to Malaprade (3).

† In these experiments the oxidation was carried out at 5°.

In sodium bicarbonate solution containing a moderate excess of oxidant, tartaric acid consumed almost 3 moles of periodate at room temperature in 20 to 30 minutes (Table I, Experiments 3 to 5). Even with glyoxylic acid in excess, rapid oxidation occurred (Experiments 1 and 2). At 5° or in strongly acid solution (Experiments 6 to 13) the secondary oxidation was slowed down considerably, while in weakly acid solution (sodium periodate solutions have a pH of about 4) intermediate effects were observed (Experiments 14 to 17).

**Carbon Dioxide Production**—When the reaction is performed with a large excess of sodium periodate, tartaric acid can be made to yield, in the Van Slyke-Neill manometric apparatus, 2 moles of carbon dioxide in 10 minutes (Table IV, Experiment 3).<sup>1</sup> The procedure described in the experimental part invariably yielded easily reproducible results. No carbon dioxide was liberated from formaldehyde and formic, glycolic, lactic, and oxalic acids under the conditions employed.

TABLE II  
*Oxidation of  $\alpha$ -Keto Acids by Periodic Acid*

Experiment No.	Substance	Consumption of periodate*			Decarboxylation	
		Initial molar ratio of oxidant to substance	Duration of oxidation	Oxidant consumed per mole of substance	Duration of oxidation	Carbon dioxide formed per mole of substance
			min.	moles	min	mole
1	Glyoxylic acid	1.8 (b)	60	0.97	10	1.0
2	Pyruvic acid	1.8 "	120	0.28	10	0.26
3	" "				30	0.53
4	Hydroxypyruvic acid†	2.1 "	30	1.0	10	0.05
5	Mesoxalic acid§	1.5 (a)	20	1.0	10	1.0
6	$\alpha$ -Ketobutyric acid	2.0 (b)	120	0.36	10	0.34
7	$\beta$ -Hydroxy- $\alpha$ -ketobutyric acid†	1.7 "	30	1.0	10	0.03
8	$\alpha$ -Ketoglutaric acid	1.8 "	120	0.28	10	0.17
9	2-Keto- <i>d</i> -gluconic acid§	5.7 (a)	90	3.5	10	0.14
10	" "	9.2 (b)	90	4.0		
11	2-Keto- <i>l</i> -gulonic acid§	5.7 (a)	90	3.4	10	0.13
12	" "	9.2 (b)	90	4.0		

\* The conditions under which the oxidations were carried out are indicated as follows: (a) = weakly acid (sodium periodate, about pH 4), (b) = weakly basic (periodic acid in excess sodium bicarbonate). For the details, also with respect to analytical procedures, see the experimental part.

† 1 cc. each of solution, of water, and of 0.43 M sodium periodate in the Van Slyke-Neill manometric apparatus.

‡ Compare the preceding paper (1) for a detailed discussion.

§ Additional data on the oxidation mechanism in the experimental part.

### *Oxidation of $\alpha$ -Keto Acids (Table II)*

The rates of the oxidative cleavage of  $\alpha$ -aldehyde and  $\alpha$ -keto acids by periodic acid revealed no definite regularity. Two compounds stood out by the readiness with which they were decarboxylated oxidatively;

<sup>1</sup> It should be clearly understood that the determinations of periodate consumption and carbon dioxide production, though placed side by side in Tables II to VII for reasons of brevity and convenience, represent two independent sets of experiments.

namely glyoxylic and mesoxalic acids.<sup>2</sup> The solution of *glyoxylic acid* employed, which was 0.3 M with respect to its aldehyde content, consumed 0.29 mole of periodic acid and produced 0.28 mole of carbon dioxide. *Mesoxalic acid*,  $\text{COOH}\cdot\text{CO}\cdot\text{COOH}$ , likewise reacted very rapidly with the consumption of 1 mole of oxidant and the formation of 1 mole each of oxalic acid and carbon dioxide. Pyruvic,  $\alpha$ -ketobutyric, and  $\alpha$ -ketoglutaric acids reacted much less readily with respect to both the consumption of periodate and the production of carbon dioxide; but it will be noted that the extent of decarboxylation of these substances still was greater than that of simple  $\beta$ -hydroxyketo acids (compare Experiments 2, 3, 6, Table II, with Experiments 4 and 7).

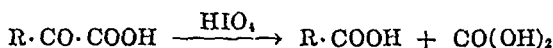
In an attempt at a general formulation of the oxidation of  $\alpha$ -keto acids by periodic acid, these compounds would, therefore, appear to behave as

TABLE III  
*Oxidation of  $\beta$ -Hydroxy- $\alpha$ -amino Acids by Periodic Acid*

Experiment No.	Substance	Consumption of periodate*			Decarboxylation*	
		Initial molar ratio of oxidant to substance	Duration of oxidation	Oxidant consumed per mole of substance	Duration of oxidation	Carbon dioxide formed per mole of substance
			<i>min.</i>	<i>moles</i>	<i>min.</i>	<i>mole</i>
1	<i>dl</i> -Serine	2.9 (a)	60	2.0	10	1.0
2	"	3.8 (b)	60	1.9		
3	<i>dl</i> -Threonine	2.9 (a)	60	2.0	10	1.0
4	"	3.8 (b)	60	2.0		

\* See Table II for the explanation.

though they were  $\alpha$ -diketones, giving rise to two acids by oxidative decarboxylation:



It is evident from what is known of this reaction that in substances possessing two or more functional groups that may compete for the oxidizing agent, *e.g.* in  $\beta$ -hydroxy- $\alpha$ -keto acids, the much more rapid rate at which the  $\alpha$ -ketol structure is attacked will make this type of cleavage prevail. Hydroxypyruvic and  $\beta$ -hydroxy- $\alpha$ -ketobutyric acids react as  $\alpha$ -ketols (1). 2-Keto-*d*-gluconic and 2-keto-*l*-gulonic acids consume 4 moles of periodate and give rise to only little carbon dioxide.

<sup>2</sup> It is noteworthy that these keto acids are known to form stable hydrates. The oxidative cleavage of  $\alpha$ -keto acids by lead tetraacetate in the presence of hydroxyl-forming substances was studied by Baer (11).

### Oxidation of $\beta$ -Hydroxy- $\alpha$ -amino Acids (Table III)

*dl*-Serine and *dl*-threonine consumed 2 moles of periodate under weakly alkaline or weakly acid conditions with the production of 1 mole of carbon dioxide from the glyoxylic acid arising from the instantaneous cleavage of the amino acids.

TABLE IV  
Oxidation of Polyhydroxy Acids by Periodic Acid

Experiment No.	Substance	Consumption of periodate*			Decarboxylation*	
		Initial molar ratio of oxidant to substance	Duration of oxidation	Oxidant consumed per mole of substance	Duration of oxidation	Carbon dioxide formed per mole of substance
			<i>min.</i>	<i>moles</i>	<i>min.</i>	<i>moles</i>
1	Glyceric acid	3.5 (a)	60	2.0	10	1.0
2	" "	4.4 (b)	60	1.9		
3	Tartaric "†	4.3 "	60	3.0	10	2.0
4	Dihydroxymaleic acid‡	3.2 "	10	2.0		
5	<i>d</i> -Gluconic acid	7.3 "	60	5.0	10	1.0
6	5-Keto- <i>d</i> -gluconic acid‡	5.7 (a)	90	3.6	10	0.33
7	" "	7.3 (b)	90	3.9	20§	0.73
8	<i>d</i> -Glucuronic acid‡	14.3 (a)	60	4.6	10	0.3
9	" "	7.3 (b)	60	4.9	120	0.9
10	Mucic acid	11.0 "	60	4.9	10	2.0
11	<i>d</i> -Saccharic acid	8.6 (a)	60	5.0	10	2.0

\* See Table II for the explanation.

† Compare Table I for more details on periodate consumption.

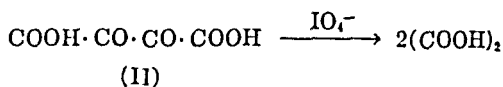
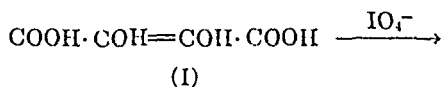
‡ Additional data on the oxidation mechanism in the experimental part.

§ 1 cc. of the solution was treated for 10 minutes with 1 cc. of sodium periodate solution. Then 1 cc. of 0.1 N sodium hydroxide was added and the reaction continued for 10 more minutes. The value is corrected for a small alkali blank.

### Oxidation of Polyhydroxy Acids (Table IV)

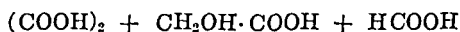
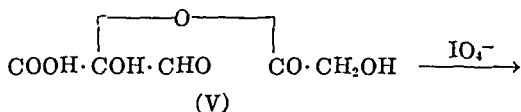
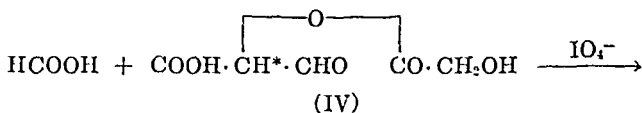
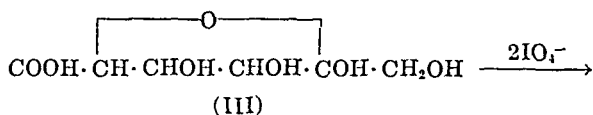
In order to avoid the complications brought about by the possible presence of lactone bridges, the neutral solutions of the polyhydroxy acids were subjected to oxidation. Glyceric, mucic, *d*-saccharic, and *d*-gluconic acids consumed oxidant and produced carbon dioxide, in accordance with the number of molecules of glyoxylic acid formed in their oxidation, and at a rate similar to that observed with tartaric acid.

*Dihydroxymaleic acid* (I) consumed 2 moles of periodate very rapidly, yielding 2 moles of oxalic acid. 1 mole of the oxidant was presumably used for the production from the enediol (I) of the dicarbonyl compound (II) and another mole for its cleavage to oxalic acid.



*5-Keto-d-gluconic acid* reacted in a manner which is difficult to interpret (Experiments 6 and 7, Table IV). It consumed about 4 moles of periodate and gave rise to no more than 0.33 mole of carbon dioxide under normal conditions. In the presence of alkali the extent of oxidative decarboxylation was increased (Experiment 7). Following an oxidation for 24 hours, when 4.25 moles of periodate had been consumed, 0.18 mole of formaldehyde and 0.46 mole of oxalic acid were produced. If this keto acid is formulated as having the open chain structure, it should have reacted either via a trihydroxyglutaric acid, consuming a total of 5 moles of oxidant to give rise to 1 mole of formaldehyde and 2 moles of carbon dioxide, or by the way of a cleavage to glycolic acid and tartaric acid semialdehyde, in which case the consumption of 4 moles of periodate should have been accompanied by the formation of 1 mole of carbon dioxide.

The experimental results, especially the production of oxalic acid, are in better agreement with the assumption that 5-keto-*d*-gluconic acid reacts in the main as a furanoid compound (III). The principal pathway of oxidative degradation, which entails the formation of oxalic acid, could be formulated as proceeding through a series of esters (IV, V). The postulated replacement of the active hydrogen (marked with an asterisk) in the glycolic acid ester (IV) by a hydroxyl is in agreement with the recent



observations of Huebner *et al.* (12) and with analogous findings of Hockett and collaborators in oxidation experiments with lead tetraacetate (13).

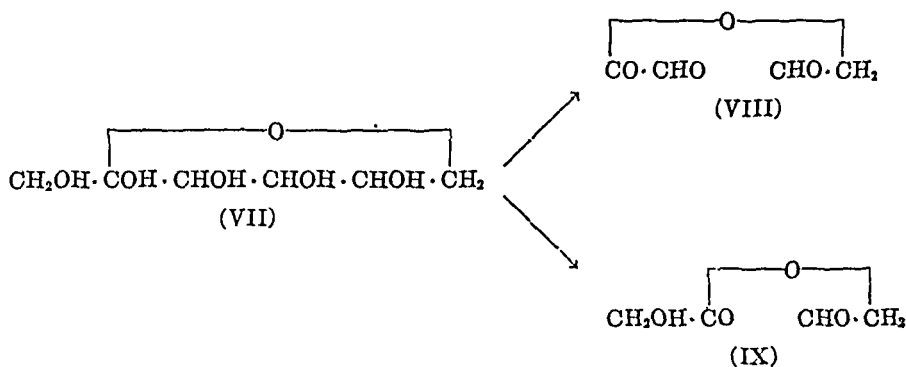
*d-Glucuronic acid* (VI) reduced about 5 moles of periodate and produced





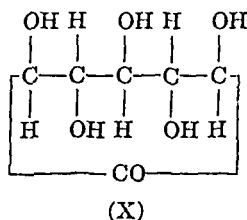
same period if the oxidation is carried out in bicarbonate solution. Almost no decarboxylation is observed under ordinary conditions, even by prolonged oxidation. In the presence of weak alkali, however, 0.7 and 0.8 moles of carbon dioxide are produced from fructose and sorbose respectively (Experiments 2 and 3, Table V). The production from fructose of 0.7 mole of carbon dioxide is in good agreement with the isolation of 1.7 moles of formaldehyde (14).

The various findings can best be reconciled if it is assumed that the major part (70 to 80 per cent) of the keto sugar (VII) is oxidized to the glyoxylic acid ester (VIII), which then gives rise to carbon dioxide. A possible side reaction (20 to 30 per cent of the ketose), involving the intermediary formation of a glycolic acid ester (IX), would explain the deficits observed



in the production of both carbon dioxide and formaldehyde and in the total consumption of periodate.

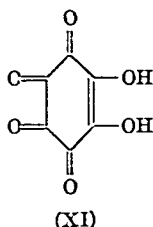
*meso-Inosose* (X) reduced 6 moles of periodate rapidly in bicarbonate



solution; in weakly acidic solution the reaction was less complete. The production of carbon dioxide (0.8 mole in 1 hour) was not affected by the addition of alkali.

#### *Oxidation of Polycarbonyl Compounds (Table VI)*

*Rhodizonic acid* (XI) consumed 4.35 moles of the oxidant and yielded 2.3 moles of oxalic acid and 1.15 moles of carbon dioxide. The very rapid reaction was accompanied by the instantaneous loss of color. The relative



proportions of oxalic acid and carbon dioxide isolated and the amount of oxidant used are in agreement with the assumption that the intermediate

TABLE VI  
*Oxidation of Polycarbonyl Compounds by Periodic Acid*

Experiment No.	Substance	Consumption of periodate*			Decarboxylation*	
		Initial molar ratio of oxidant to substance	Duration of oxidation	Oxidant consumed per mole of substance	Duration of oxidation	Carbon dioxide formed per mole of substance
1	Dipotassium rhodizonate†	6.5 (a)	min. 90	moles 4.35	min. 10‡	moles 1.15
2	" "				30‡	1.15
3	Dipotassium croconate†	5.8 "	90	4.0	10	1.03
4	" "	7.3 (b)	90	4.1	20	1.03
5	Triketohydrindene hydrate	2.9 (a)	10	1.0	10	0
6	" "	2.9 "	60	1.0	20	0
7	" "	2.9 (b)	60	1.0		

\* See Table II for the explanation.

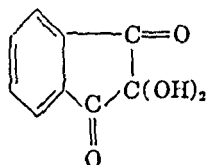
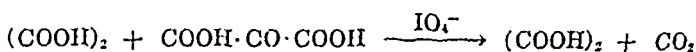
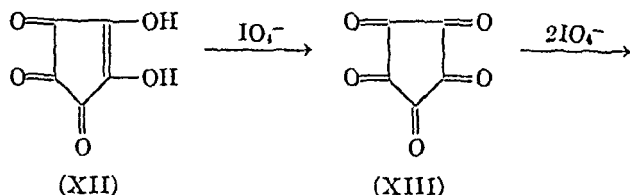
† Additional data on the oxidation mechanism in the experimental part.

‡ The potassium rhodizonate powder (0.02 mm) was weighed into the cup of the manometric apparatus, washed into the chamber with 2 cc. of water, and oxidized with 1 cc. of 0.43 M sodium periodate in the usual manner.

triquinoyl is further degraded in two ways; namely, partly through mesoxalic acid to oxalic acid and carbon dioxide, partly to oxalic acid directly.

*Croconic acid* (XII) reduced 4 moles of periodate and yielded 1.9 moles of oxalic acid and 1.03 moles of carbon dioxide. As in the reaction scheme previously discussed for compound I, the enediol apparently first consumed 1 mole of oxidant, accompanied by the fading of the yellow color of XII, to form leuconic acid (XIII), which then was further degraded via mesoxalic acid.

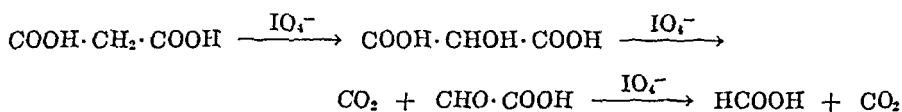
*Triketohydrindene hydrate* (XIV) consumed exactly 1 mole of periodate; no carbon dioxide was formed in the reaction. The oxidation presumably stopped with the formation of phthalonic acid.



(XIV)

### *Oxidation of Malonic and Tartronic Acids (Table VII)*

The treatment of these acids with periodate resulted in the production from both compounds of 2 moles of carbon dioxide in 2 hours. The consumption of periodate proceeded rather slowly. *Malonic acid* reduced 3 moles of the oxidant, *tartronic* (hydroxymalonic) *acid* 2 moles. Neither compound formed oxalic acid. A possible reaction mechanism could be based on the assumption that the replacement of an active hydrogen by a hydroxyl (12) resulted in the conversion of malonic to tartronic acid, which then was further degraded via glyoxylic acid.



The findings presented in the preceding pages show the liberation of carbon dioxide in the course of oxidations with periodate to be an indication of the presence, or the formation, of glyoxylic or mesoxalic acid in the oxidation mixture. With many compounds the results are entirely unequivocal. Difficulties of interpretation are, however, encountered occasionally in substances, such as the keto sugars, possessing stable oxygen bridges in which the esters resulting from the primary attack of the oxidant may be susceptible of multiple secondary degradation. Much additional work along these lines obviously is necessary. With respect to the detection of glyoxylic acid formed by the action of periodic acid it should be understood that the concentration of periodate will, in certain cases, affect the rate of oxidation considerably. For instance, in the investigation of compounds, such as 2,3-dimethylmannosaccharic acid (15) or 2,3-dimethylmucic acid (16), that require only 1 mole of, and react rapidly with, periodic acid, the glyoxylic acid which is produced appears to remain

unattacked in the absence of an excess of periodate. This is especially true of lactones that give rise to stable esters of glyoxylic acid following oxidation with periodate (17, 18).

## EXPERIMENTAL

*Material*

The compounds listed here are given in the order in which they appear in Tables II to VII. All other substances were commercially available pure preparations.

*Glyoxylic acid* was prepared in solution according to Benedict (19) by the reduction of oxalic acid by magnesium powder, followed by the extrac-

TABLE VII  
*Oxidation of Malonic and Tartronic Acids*

Experiment No.	Substance	Consumption of periodate*			Decarboxylation†	
		Initial molar ratio of oxidant to substance	Duration of oxidation	Oxidant consumed per mole of substance	Duration of oxidation	Carbon dioxide formed per mole of substance
1	Malonic acid	2.9 (a)	180	1.2	120	1.93
2	" "	5.8 "	1140	3.1		
3	Tartronic acid	4.3 "	120	1.5	60	1.90
4	" "	4.3 "	240	1.6	90	1.96
5	" "	5.1 "	1140	2.0		

\* See Table II for the explanation.

† 1 cc. of solution and 2 cc. of 0.43 M sodium periodate in the Van Slyke-Neill manometric apparatus.

tion of the reaction mixture with ether. The resulting solution was 0.66 M with respect to total acid content (glyoxylic and glycolic acids) and 0.3 M with respect to aldehydic acid content (20). We are indebted to Dr. P. K. Stumpf for this solution.

The preparation of *hydroxypyruvic*,  $\alpha$ -*ketobutyric*, and  $\beta$ -*hydroxy- $\alpha$ -ketobutyric acids* has been discussed in the preceding paper (1).

*Mesoxalic acid* was obtained through the courtesy of Dr. D. E. Green.  $\alpha$ -*Ketoglutaric acid*, m.p. 113–114°, was synthesized by a method soon to be presented by Martell and Herbst (private communication).

*2-Keto-d-gluconic acid*, employed as the sodium salt, was prepared from the corresponding calcium salt trihydrate.<sup>3</sup>

<sup>3</sup> We are highly indebted to Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc., Nutley, New Jersey, for these specimens, as well as for samples of 2-keto-l-gulonic and d-gluconic acids, potassium acid d-saccharate, and l-sorbose.

*Sodium 5-keto-d-gluconate* was prepared from the calcium salt ( $2\frac{1}{2}\text{H}_2\text{O}$ )<sup>4</sup> by prolonged shaking with the required amount of sodium oxalate. The conversion was complete, as was shown by the weight and oxidimetric titration value of the resulting calcium oxalate.

*meso-Inosose* was prepared by the action of *Acetobacter suboxydans* (American Type Culture Collection No. 621) on *meso*-inositol (21, 22) and purified through its phenylhydrazone (22), m.p. 182° (with decomposition). The purified *meso*-inosose melted with decomposition at 199–200°.

*Dipotassium rhodizonate* was prepared from *meso*-inositol (23).

$\text{C}_6\text{O}_6\text{K}_2$  (246.3). Calculated, C 29.3, K 31.8; found, C 29.0, K 32.1

*Dipotassium croconate* was synthesized from rhodizonic acid (24).

*Tartronic acid* was prepared from dihydroxytartaric acid (25). A suspension of 5.6 gm. of sodium dihydroxytartrate in 60 cc. of water was kept at 80° with stirring for 1 hour, when complete solution had taken place. The calcium tartronate, precipitated with 24 cc. of  $\text{M}$  calcium chloride, had a dry weight of 3.0 gm. The solution of the dry material in 6 cc. of 20 per cent hydrochloric acid was extracted eight times with 20 cc. portions of ether (26). 1 gm. of crude tartronic acid was recovered from the ethereal solution; recrystallization from absolute ether yielded 0.3 gm. of tartronic acid, melting at 160–162° (with decomposition); molecular weight (by titration) 116; required by  $\text{C}_3\text{H}_4\text{O}_5$ , 120.

### Reagents

Nearly 0.54  $\text{M}$   $\text{HIO}_4$  solutions were prepared by dissolving 12.5 gm. of crystalline  $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$  per 100 cc. of solution. A similar amount of nearly 0.43  $\text{M}$   $\text{NaIO}_4$  was prepared from 9.5 to 10.0 gm. of sodium metaperiodate.<sup>4</sup> The solutions were allowed to stand overnight or longer and filtered on a fritted glass funnel to remove a small precipitate. They were then standardized against 0.1  $\text{N}$  arsenite solution.

### Determination of Periodate Consumption

The procedures followed in the oxidation of tartaric acid are described in Table I.

In experiments with other substances (Tables II to VII, in which conditions and amounts employed in each case are indicated) 1 to 10 cc. of a 0.01 to 0.03  $\text{M}$  solution were treated by one of the two following methods. In procedure (a) the oxidation was carried out with 0.43  $\text{M}$  sodium periodate in the presence of 15 cc. of water; then a few cc. of  $\text{M}$  sodium bicarbonate

<sup>4</sup> Paraperiodic acid and sodium metaperiodate were obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio.

were added, followed immediately by 1 cc. of 20 per cent potassium iodide. In procedure (b) after oxidation with 0.5*M* periodic acid in the presence of 15 cc. of *M* sodium bicarbonate 1 cc. of 20 per cent potassium iodide was added. The liberated iodine was, in both procedures, titrated with 0.1 *N* arsenite (27, 28).

#### *Determination of Carbon Dioxide Production*

1 cc. of solution (0.03 or 0.01 *M* when 1 or 2 moles of carbon dioxide respectively are liberated) is measured into the chamber of the Van Slyke-Neill manometric apparatus. This is followed by 1 cc. of water and 1 cc. of NaIO<sub>4</sub> solution. The mercury is lowered to the bottom of the chamber, returned slowly into the chamber, and then lowered to the 50 cc. mark. After being shaken for 15 seconds, the chamber contents are allowed to stand for the stated time. The CO<sub>2</sub> is now liberated with 1 cc. of 2 *N* acetic acid,<sup>5</sup> absorbed by 0.5 cc. of 5 *N* NaOH, and the *P*<sub>CO<sub>2</sub></sub> determined in the usual manner (29, 30). Mg. of carbon were calculated by multiplying by the factors given in (28). The values so obtained were used to calculate the moles of CO<sub>2</sub>, which are reported in Tables II to VII. Although these factors have been arrived at under somewhat different experimental conditions, their adequacy for the present purpose was demonstrated in experiments with tartaric acid as a test substance. Each experiment was carried out at least in duplicate. The results invariably showed very good agreement.

#### *Additional Data on Oxidation Mechanisms; Determination of Oxalic Acid and of Formaldehyde*

*Determination of Oxalic Acid*—For the determination of *oxalic acid* an aliquot of the oxidation mixture was acidified with 2 *N* hydrochloric acid, and 7.5 *M* hydriodic acid was added (31) in an amount sufficient to reduce both the remaining periodic and the formed iodic acids. Iodine was removed by filtration and, from the filtrate, by the necessary amount of *M* sodium thiosulfate. From the solutions, neutralized with concentrated ammonia followed by acidification (to methyl red) with glacial acetic acid, oxalic acid was precipitated as the calcium salt. This salt then was either analyzed both gravimetrically (as the monohydrate) and oxidimetrically (with standard potassium permanganate) or dissolved directly in 2 *N* sulfuric acid and subjected to a permanganate titration.

*Determination of Formaldehyde*—An aliquot of the oxidation mixture was made alkaline with sodium bicarbonate and unused periodate destroyed with 2 *M* sodium arsenite. The solution was made acid to methyl red with acetic acid and the formaldehyde precipitated and estimated as

<sup>5</sup> The use of 2 *N* acetic acid was found preferable to other methods of acidification.

the dimedon derivative, a 0.4 per cent solution of the reagent being used (32). Removal of both iodate and periodate with arsenite under acidic conditions (14) gave the same result.

*Mesoxalic Acid (Experiment 5, Table II)*—To 20 cc. of a 0.03 M solution of sodium mesoxalate,  $C_3H_2O_6Na_2$ , 3 cc. of water and 2 cc. of 0.43 M sodium periodate were added. After 20 minutes, a total consumption of 0.6 mM of periodate was found, as shown by the titration of a 2 cc. aliquot. The production of *oxalic acid* amounted to 0.597 mM.

*2-Keto-d-gluconic and 2-Keto-l-gulonic Acids (Experiments 9 to 12, Table II)*—2 mM of the sodium salts were oxidized with 25 cc. of 0.43 M sodium periodate in a final volume of 100 cc. After 20 hours the consumption of oxidant, as was shown by titration of a 5 cc. aliquot, amounted to 3.5 moles per mole of keto acid; i.e., the same value as that shown after 90 minutes (Table II, Experiments 9 and 11). The addition of 2 gm. of sodium bicarbonate caused the oxidation to proceed to the 4 mole level in 1 additional hour, after which 2-keto-d-gluconic acid gave rise to 0.82 mole and 2-keto-l-gulonic acid to 0.85 mole of *oxalic acid*. Exactly 1 mole of formaldehyde per mole of keto acid was isolated as the dimedon derivative (m.p. and mixed m.p. 190–192°).

*Dihydroxymaleic Acid (Experiment 4, Table IV)*—To a solution of 148 mg. of the dihydrate,  $C_4H_4O_6 \cdot 2H_2O$  (0.8 mM), in 35 cc. of water and 5 cc. of M sodium bicarbonate, 6 cc. of 0.43 M sodium periodate were added dropwise, in order to avoid the liberation of iodine. The mixture was adjusted to a volume of 50 cc. The titration of 5 cc. aliquots, removed 10 and 30 minutes after the start of the experiment, showed in both cases a total consumption of periodate corresponding to 1.6 mM. The remainder of the oxidation mixture served for the isolation of *oxalic acid*, which was obtained in an amount corresponding to a total production of 1.54 mM.

*5-Keto-d-gluconic Acid (Experiments 6 and 7, Table IV)*—An aqueous suspension of 0.944 gm. of calcium 5-keto-d-gluconate ( $2\frac{1}{2}H_2O$ ) was warmed slightly with a solution of 254 mg. of oxalic acid dihydrate. After 5 hours with occasional shaking the calcium oxalate was filtered off and 336 mg. of sodium bicarbonate were added to the filtrate, which had a volume of about 100 cc. Following the addition of 50 cc. of a 0.43 M sodium periodate solution, the volume of the mixture was adjusted to 200 cc. The titration of a 5 cc. aliquot 24 hours later showed that 4.25 moles of oxidant had been consumed per mole of keto acid. (At this point no change in periodate consumption was observed when a portion of the solution was made alkaline for an additional 90 minutes by the addition of an equal volume of M sodium bicarbonate.) For the determination of the *oxalic acid* formed, 100 cc. of the oxidation mixture were treated as described above. The calcium oxalate monohydrate (0.141 gm.) required 18.2 cc. of 0.1 N  $KMnO_4$ .



1 mole of the keto acid, therefore, yielded 0.46 mole of oxalic acid. From a 75 cc. aliquot formaldehyde was isolated as the dimedon derivative, melting at 189–191°. The production of formaldehyde amounted to 0.18 mole per mole of keto acid.

In view of the probable formation of glycolic acid as one of the oxidation products of 5-keto-*d*-gluconic acid, which was discussed before, it should be mentioned that glycolic acid, both in weakly acid and in alkaline solution, proved inert to periodate.

*d*-Glucuronic Acid (Experiments 8 and 9, Table IV)—The oxidation of 1 mM of sodium glucuronate with 15 cc. of 0.425 *M* NaIO<sub>4</sub> in a total volume of 50 cc. for 24 hours showed a consumption of 5.09 moles of oxidant per mole of acid. The same value was obtained on an aliquot which was treated for 1½ hours longer with an equal volume of *M* sodium bicarbonate. The analysis of a 40 cc. aliquot gave 61 mg. of calcium oxalate monohydrate, requiring 8.0 cc. of 0.1 *N* KMnO<sub>4</sub>, i.e. 0.5 mole of oxalic acid per mole of glucuronic acid.

Dipotassium Rhodizonate (Experiments 1 and 2, Table VI)—A total of 1 mM (0.246 gm.) of the salt was mixed in small portions with 90 cc. of 0.07 *M* sodium periodate in such a manner as to avoid too great a temporary excess of either oxidant or substrate. After about one-third of the rhodizonate had been stirred into 30 cc. of oxidant, another 30 cc. portion of periodate was added and this procedure was repeated once more. The color of the salt faded immediately on contact with the oxidant. The mixture was brought to a volume of 100 cc.; the titration of 5 cc. aliquots after 1½, 4½, and 24 hours showed a consumption of a total of 4.35 to 4.40 mM of periodate. Oxalic acid, produced in this oxidation experiment, amounted to 2.3 mM. In another experiment with 1 mM of dipotassium rhodizonate 2.15 mM of oxalic acid were isolated.

Dipotassium Croconate (Experiments 3 and 4, Table VI)—To an aqueous solution of 109 mg. (0.5 mM) of the salt 5 cc. of 0.43 *M* NaIO<sub>4</sub> were added and the mixture was adjusted with water to a volume of 50 cc. 3 hours later, the titration of a 5 cc. aliquot indicated the consumption of 3.9 moles of oxidant per mole of substrate. The determination of oxalic acid showed the production of 1.9 moles per mole of the salt.

#### SUMMARY

The conditions and products of the oxidative decarboxylation by periodic acid of  $\alpha$ -keto,  $\beta$ -hydroxy- $\alpha$ -amino, and polyhydroxy acids, ketoses, polycarbonyl compounds, and malonic and tartronic acids are discussed in detail.

Outstanding in the speed of oxidation are glyoxylic and mesoxalic acids, which are decarboxylated quantitatively in 10 minutes. Compounds

giving rise to these keto acids after oxidation with periodate likewise produce the theoretical amount of carbon dioxide.

Polycarbonyl compounds possessing an enediol structure (rhodizonic and croconic acids) similarly react with periodate, most likely with the intermediary formation of mesoxalic acid.

The results obtained with malonic and tartronic acids agree with the assumption that malonic acid first is hydroxylated to tartronic acid, which then is oxidized to glyoxylic acid and carbon dioxide. Tartronic acid is the only monohydroxy acid encountered which is oxidized.

The influence exerted by stable oxygen bridges is exemplified by the apparent multiplicity of mechanisms underlying the oxidative degradation of *d*-glucuronic acid and of keto sugars.

*d*-Glucuronic acid yields carbon dioxide and oxalic acid. It probably reacts through several pathways, partly by the way of glyoxylic acid as an intermediate, partly via hydroxylation of the hydrogen next to the carboxyl.

5-Keto-*d*-gluconic acid produces small amounts of formaldehyde and carbon dioxide and about 0.5 mole of oxalic acid, results best reconciled by the postulation of a furanoid structure for this compound.

Keto sugars do not yield carbon dioxide, except after alkalization of the oxidation mixture. Reaction mechanisms, which take into account the consumption of periodate and the production of carbon dioxide and formaldehyde, are discussed.

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# DEGRADATION OF THE BILE ACID SIDE CHAIN\*

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Preliminary experiments conducted 2 years ago indicated the feasibility of converting desoxycholic acid into  $3\alpha,12\beta$ -dihydroxyetiocholanone-17 by comparatively simple degradative operations. The first consisted in allylic bromination of the intermediate  $3,12$ -diacetoxydiphenylcholene (I) by the Ziegler method, dehydrohalogenation to the diphenylcholadiene, and oxidation to  $3\alpha,12\beta$ -diacetoxyetiocholanyl methyl ketone. This method was communicated on March 11, 1944, to the cooperating group, but in the meantime Meystre, Miescher, and coworkers (2) have anticipated our publication and have described (3) an improved procedure that constitutes a highly practical method of degradation. The second operation consisted in the conversion of the free 20-methyl ketone to  $3\alpha,12\beta$ -dihydroxyetiocholanone-17 by nitrite cleavage (4) and hydrolysis of the resulting oxime.

## EXPERIMENTAL

*3 $\alpha,12\beta$ -Dihydroxyetiocholanyl Methyl Ketone (III)*—A summary of the best result is as follows: A solution of 25 gm. of  $3\alpha,12\beta$ -diacetoxy-24,24-diphenyl- $\Delta^{23}$ -cholene (I, m.p. 160.5–161.5°) and 7.5 gm. of N-bromosuccinimide in 125 cc. of benzene was refluxed for 2 hours, the mixture was cooled and filtered, and the product recovered from the filtrate heated with collidine for 4 hours at 130°. Crystallization of recovered product from acetone-methanol gave 8.5 gm. of I, m.p. 159–160.5°, and 14.2 gm. of crude  $3\alpha,12\beta$ -diacetoxy-24,24-diphenyl- $\Delta^{20-22,23}$ -choladiene (II). Purified mixtures of I and II were characterized by analysis (C 80.99, H 8.41) and absorption spectrum (maximum 305 m $\mu$ ). The crude diene was ozonized in methanol-ethyl acetate, the ozonide decomposed with zinc dust and acetic acid, and the recovered material treated with chromic acid in acetic acid at room temperature overnight. The neutral fraction (9.5 gm.) was refluxed with 5 per cent methyl alcoholic potassium hydroxide, neutralized with acetic acid, steam-distilled to remove benzophenone, and the ketonic material was separated with Girard's Reagent T and crystallized from ether, with recovery of material from the mother liquor by chromatography. The total yield of the ketone III, crystallized finally

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

from dilute methanol and melting at 171–173°, was 2.1 gm. (23 per cent). An earlier sample crystallized from ether and from ethyl acetate-ligroin melted at 174.5–177° and did not depress the melting point of an authentic sample (m.p. 178–180°) kindly provided by Dr. R. B. Turner.

*Analysis*— $C_{21}H_{34}O_2$ . Calculated. C 75.40, H 10.25  
Found. " 75.05, " 10.46

Further identification was made (mixed melting point) of the corresponding diketone (m.p. 195–199°) and diacetate (m.p. 140–141°).<sup>1</sup>

*3 $\alpha$ ,12 $\beta$ -Dihydroxyetiocholanone-17 (IV)*—A solution of 334 mg. of III in 2 cc. of absolute alcohol was treated with 0.7 cc. of a solution of 0.69 gm. of sodium in 20 cc. of absolute alcohol, and 0.5 cc. of ethyl nitrite was added. The mixture stood at room temperature for 1 week and then was neutralized with carbon dioxide, filtered, and evaporated in a vacuum. The residue, which gave a red color test with ferric chloride in alcohol, was hydrolyzed by being refluxed for 3 hours with 1 cc. of concentrated hydrochloric acid in 5 cc. of 50 per cent alcohol. The solution was diluted with water and the product recovered by ether extraction. The residue (0.17 gm.) was treated with Girard's Reagent T and the water-soluble product submitted to gentle hydrolysis according to Reich and Reichstein (5), when 0.03 gm. of starting material IV was recovered. Further hydrolysis in the presence of added hydrochloric acid gave 0.07 gm. of product (m.p. 142–148°) that reduced Tollens' reagent. The material was chromatographed on alumina. The product recovered by elution with ether (1 part)-methanol (19 parts) afforded 59 mg. (20 per cent) of crude IV, m.p. 157–162°, which did not reduce Tollens' reagent. Further purification through the Girard derivative and crystallization from ether afforded pure material melting at 164–165° (Reich (6) 166–167°).

*Analysis*— $C_{19}H_{30}O_3$ . Calculated. C 74.47, H 9.87  
Found. " 74.27, " 9.93

The diacetyl derivative had the constants, m.p. 157.5–158.5°,  $\alpha_D^{27} = +179^\circ$  in acetone (Reich (6), 160–161°,  $\alpha_D^{17} = +178^\circ$  in acetone).

*Analysis*— $C_{23}H_{34}O_5$ . Calculated. C 70.74, H 8.78  
Found. " 70.93, " 9.17

#### SUMMARY

Degradation of the side chain of desoxycholic acid is accomplished by allylic bromination of 3,12-diacetoxydiphenylcholene, conversion to the

<sup>1</sup> The diacetate was obtained by crystallization from petroleum ether as a polymorph melting 20° higher than previously recorded for this compound.

diphenylcholadiene, and oxidation to 3 $\alpha$ ,12 $\beta$ -diacetoxyetiocholanyl methyl ketone; the ketone on nitrite cleavage and hydrolysis affords 3 $\alpha$ ,12 $\beta$ -dihydroxyetiocholanone-17.

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# THE FIXATION OF CARBON DIOXIDE IN OXALACETATE BY PIGEON LIVER\*

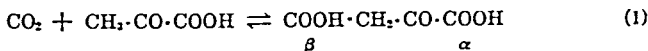
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A preliminary report on the fixation of  $C^{14}O_2$  in oxalacetate by pigeon liver extracts has been presented previously (1). This communication is a more detailed account and deals particularly with the effect of adenosine triphosphate upon this reaction.

Wood and Werkman (2) first postulated Reaction 1 as a mechanism of  $CO_2$  fixation when studying the fermentation of glycerol by propionic acid bacteria.



It was noted that in this fermentation a stoichiometric relationship existed between succinate formed and  $CO_2$  utilized, and since pyruvate had previously been shown to be an intermediate in the glycerol fermentation (3), Reaction 1, coupled with a reduction of oxalacetate to succinate, was proposed to account for the formation of succinate from glycerol and  $CO_2$ .

Krampitz *et al.* (4) gave the first direct proof of the reversibility of Reaction 1 by their studies with a bacterial enzyme preparation which decarboxylated oxalacetate to pyruvate and  $CO_2$  (5). By carrying out this reaction in the presence of  $NaHC^{14}O_3$ , it was possible to show that the carboxyl  $\beta$  to the carbonyl group of the residual oxalacetate contained excess  $C^{14}$ , and thus that the reaction was reversible.

Despite ample evidence of  $CO_2$  fixation by animal tissues (6-8), direct demonstration of Reaction 1 with these preparations was not accomplished, although it was tacitly assumed in most cases that such a mechanism was involved. Evans *et al.* (9) described an extract from an acetone powder of pigeon liver, which fixed  $C^{14}O_2$  in organic combination, when fumarate was fermented in the presence of pyruvate and  $NaHC^{14}O_3$ . Wood, Venesland, and Evans (10) later showed that the  $CO_2$  fixed in this system was distributed almost equally among the carboxyl groups of pyruvate, lactate, fumarate, and malate. Although this type of distribution could be explained via Reaction 1 in conjunction with other well established

\* Aided by grants from The National Foundation for Infantile Paralysis, Inc., and the Rockefeller Foundation, Research in Biology and Medicine.

reactions, an attempt by Wood *et al.* (10) to demonstrate exchange between oxalacetate, as such, and isotopic bicarbonate failed. However, it has now been possible to show that, when adenosine triphosphate is added to the reaction mixture, a rapid exchange of isotopic carbon does occur.

Although this finding demonstrates a fixation of  $\text{CO}_2$  in oxalacetate in pigeon liver, the relative importance of this reaction in the over-all fixation of  $\text{CO}_2$  by animals (8) and by animal tissue (6, 7) is not established. Primary reactions for fixation of  $\text{CO}_2$  involving two other compounds have been reported: (a) oxalosuccinate (Ochoa (11)) and (b) pyruvate (Lipmann and Tuttle (12), Utter *et al.* (13)). The oxalosuccinate reaction was demonstrated with a preparation from pig heart and the pyruvate reaction in a bacterial preparation.

### *Methods and Materials*

*Enzyme Preparation*—The liver preparations used in these experiments were obtained by a method similar to that of Evans *et al.* (9), but with some variations. The procedure was as follows: Livers were rapidly removed from several pigeons after decapitation and were immediately cooled. The livers were then minced in 5 volumes of cold acetone with a Waring blender with sharpened blades. After mincing for a few seconds at high speed, the preparation was stirred at a slower speed for 3 minutes. The speed of the blender was controlled by a voltage divider. The acetone was removed from the liver brei by filtration through a sintered glass Büchner funnel and the acetone treatment was repeated once. After drying *in vacuo*, the larger pieces of connective tissue were removed and the material was converted to a powder by gentle grinding in a mortar. This preparation is stable for several weeks if stored in the cold *in vacuo*.

Extracts were prepared from the powder by mixing thoroughly with 10 volumes of distilled water, incubating at  $38^\circ$  for 5 to 10 minutes, and centrifuging. The centrifugate was then further treated in most cases by dialysis against distilled water or 0.9 per cent KCl. In some cases, the centrifugate was first precipitated, as described by Evans *et al.* (9), with 67 per cent  $(\text{NH}_4)_2\text{SO}_4$ , and the precipitate was taken up in water and dialyzed. Dialysis was carried out for 3 to 4 hours at  $8-12^\circ$  in thin collodion tubes which were rotated in about 20 liters of liquid, so as to give stirring both inside and outside the bag.

*Other Materials*—Unless otherwise stated, the materials were used as sodium salts adjusted to pH 7.0.

Diphosphopyridine nucleotide was obtained from yeast, as described by Williamson and Green (14). A biological assay with an apozymase from bakers' yeast, prepared according to Govier (15), indicated a purity of 50 to 60 per cent.

Adenosine triphosphate was isolated as the barium disalt from the skeletal muscle of rabbits under  $MgSO_4$  anesthesia by the method of Needham (16), as described by Lepage (17). The ratios of 9 minute-hydrolyzable P to total P, and of total P to ribose, were found to be theoretical and the material had a purity of almost 100 per cent when calculated as the tetrahydrate.

Adenylic acid was prepared by hydrolysis of adenosine triphosphate with  $Ba(OH)_2$ , followed by separation of the adenylic acid from adenosine di- and triphosphates and inorganic phosphate by the procedure outlined by Lepage and Umbreit (18). As calculated from the easily hydrolyzable P, total P, and ribose determinations, the adenylic acid contained 2 per cent adenosine triphosphate (or 4 per cent, if calculated as the diphosphate).

Oxalacetate was obtained by hydrolysis of the commercial diethyl ester by the general method of Krampitz and Werkman (5). The solutions of oxalacetate were prepared immediately before use by dissolving the free acid in an approximately equimolar amount of  $Na_2PO_4$  and diluting to volume. The final solution had a pH of 6.0 to 6.5.

Sodium pyruvate was obtained by neutralizing a weighed amount of pyruvic acid secured by vacuum distillation of the commercial product.

Isotopic bicarbonate was obtained by the liberation of  $C^{14}O_2$  from barium carbonate and the trapping of the liberated gas in carbonate-free NaOH in an evacuated system. Sufficient  $BaC^{14}O_3$  was used to make the alkali just colorless to phenolphthalein.

*Methods*—Inorganic P was determined colorimetrically by the Lohmann and Jendrassik modification (19) of the Fiske and Subbarow method (20). The phosphate fractions of adenosine triphosphate and phosphopyruvate were determined after conversion to inorganic phosphate by methods described in conjunction with the individual experiments. Lactic acid was determined by the colorimetric method of Barker and Summerson (21) and pyruvic and  $\alpha$ -ketoglutaric acids by the method of Friedemann and Haugen (22). Succinic acid was determined by the enzymatic technique of Krebs (23) and oxalacetic acid by the aniline citrate process, as described by Edson (24) and Greville (25).

*Method of Conducting Experiments*—The experiments with isotopic carbon were conducted in large Warburg vessels (135 ml. capacity) equipped with two side arms. A procedure somewhat analogous to that of Krampitz *et al.* (4) was followed; *i.e.*, oxalacetate was decarboxylated in the presence of  $NaHC^{14}O_3$  and the residual oxalacetate was tested for the presence of excess  $C^{14}$ . In our experiments, the enzyme preparation was placed in one side arm,  $NaHC^{14}O_3$  in the second side arm, and the other constituents in the main chamber. The constituents of the main chamber ordinarily included adenosine triphosphate (ATP),  $MnCl_2$ , and pyruvate. It will be

shown in Tables I and IV that the first two of these substances are necessary for rapid exchange of  $C^{13}$  between  $NaHC^{13}O_3$  and oxalacetate, while pyruvate has little effect. After gassing with nitrogen and equilibrating, the contents of the cup were mixed. Because of the acidity of the oxalacetate-phosphate mixture,  $C^{13}O_2$  was liberated from the  $NaHC^{13}O_3$ , giving an atmosphere of  $C^{13}O_2$  and nitrogen and a pH of 7.2 to 7.3. In a few experiments in which oxalacetate was neutralized with NaOH, or in which no oxalacetate was included, phosphate buffer of pH 6.2 was used to acidify the bicarbonate.

After 45 minutes at  $38^\circ$ , an aliquot was removed from the vessel for the determination of residual oxalacetate and immediately thereafter the reaction was stopped by the addition of 10 N  $H_2SO_4$ , 50 per cent citric acid, or 10 per cent  $HPO_3$ , as is indicated in the individual experiments. After removal by centrifugation of the precipitated protein, a few drops of caprylic alcohol were added as an antifoaming agent, and the  $C^{13}O_2$  was removed by rapid passage of normal tank  $CO_2$  through the solution for 10 minutes. After this process, the layer of  $CO_2$  immediately above the solution was cleared away by a blast of air, and the smaller amount of  $CO_2$  remaining in the solution was collected in a carbonate-free sodium hydroxide solution by aeration with  $CO_2$ -free air for 15 minutes. This "rinse" fraction serves to check the efficiency of the removal of the  $C^{13}O_2$ . After removal of the "rinse" fraction, a second fraction representing the  $\beta$ -COOH of oxalacetate was obtained by boiling and aerating the solution for 10 to 15 minutes and collecting the  $CO_2$  liberated by this process. The oxalacetic acid is broken down to pyruvic acid and carbon dioxide by this "acid-heat" treatment. The  $\alpha$ -carboxyl of oxalacetate, together with the carboxyl of pyruvate, was next secured by treatment of the solution with an excess of a solution of  $Ce(SO_4)_2$  and continuing the heating and aeration for 15 minutes. The pyruvate is oxidized to carbon dioxide and acetic acid by the  $Ce(SO_4)_2$  (26).

*Determination of Isotope Concentration*—The  $C^{13}$  content of the samples was determined with the aid of a Nier type mass spectrometer (27). The results are calculated as atom or mole per cent  $C^{13}$ .

$$\text{Atom \% } C^{13} = \frac{\text{moles } C^{13}}{\text{moles } C^{13} + \text{moles } C^{12}} \times 100$$

The spectrometer readings were adjusted to give a value of 1.09 per cent  $C^{13}$  for standard  $BaCO_3$  and the data in the tables are expressed as per cent of  $C^{13}$  in excess of this normal complement.

### Results

*Effect of Adenosine Triphosphate upon Fixation of  $C^{13}O_2$  in Oxalacetate*—In the experiments by Wood *et al.* (10), which failed to demonstrate the

exchange of  $C^{14}O_2$  with oxalacetate, the enzyme system was partially purified by ammonium sulfate fractionation and dialysis, and no additions other than oxalacetate, pyruvate,  $MnCl_2$ , and bicarbonate were made. In other experiments with the same enzyme preparation, fixation of isotopic  $CO_2$  was obtained with fumarate and pyruvate as the substrates, but impure diphosphopyridine nucleotide (DPN) or tissue extract was added to facilitate the reaction. Accordingly, the presence of the hydrogen carrier (DPN), as well as the oxidation-reduction reaction between fumarate and

TABLE I  
*Effect of Adenosine Triphosphate (ATP) on Fixation of  $C^{14}O_2$  in Oxalacetate by Pigeon Liver Extracts*

Experiment No	Extract	ATP added	Oxalacetate added	$\beta$ COOH of oxalacetate			Ce(SO <sub>4</sub> ) <sub>2</sub> fractions			Rinse	
				Recovered*	Excess $C^{14}$	$C^{14}$ fixed	Recovered*	Excess $C^{14}$	$C^{14}$ fixed	Recovered*	Excess $C^{14}$
		mm	mm	mm	per cent	mm $\times 10^{-4}$	mm	per cent	mm $\times 10^{-4}$	mm	per cent
1 a	Purified†		0.68	0.23	0.04	0.9	1.37	0.00	0.0	1.39	0.01
1 b	"	0.014	0.68	0.20	0.22	4.4	0.97	0.02	0.2	1.13	0.01
2 a	Crude		0.68	0.39	0.07	2.7	0.95	0.01	1.0	2.01	0.02
2 b	"	0.007	0.68	0.32	0.25	8.0	0.91	0.01	0.9	3.98	0.01
3 a	"		0.68	0.39	0.26	10.2	1.19	0.08	9.5	3.83	0.01
3 b	"	0.014	0.68	0.34	0.35	12.3	1.06	0.10	10.6	2.99	0.00

\* mm represent amounts recovered during treatment, as determined by the weight

† Extract purified by precipitation with two thirds saturated  $(NH_4)_2SO_4$  and 4 hours dialysis of the dissolved precipitate against distilled water. The additions were as follows: Experiments 1 a and 1-b, 4 ml of liver extract, 0.5 mm of Na pyruvate, 0.04 mm of  $MnCl_2$ , 0.3 mm of phosphate (pH 6.2), 0.6 mm of  $NaH^{14}CO_3$  ( $C^{14}$  = 5.1 per cent excess) in a total volume of 14 ml, time, 45 minutes; Experiments 2 a and 2 b, 3 ml of liver extract, 0.4 mm of Na pyruvate, 0.05 mm of  $MnCl_2$ , 0.4 mm of phosphate (pH 6.2), 0.7 mm of  $NaH^{14}CO_3$  ( $C^{14}$  excess = 4.6 per cent) in a total volume of 15 ml, time, 45 minutes; Experiments 3 a and 3-b, 4 ml of liver acetone extract, otherwise the same as Experiments 2 a and 2-b.

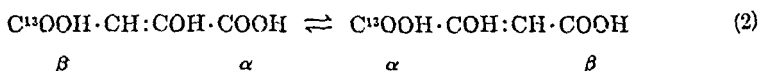
pyruvate, made the conditions of the two experiments quite dissimilar. Since the fixation of  $CO_2$  in oxalacetate has been calculated (9) to be an endergonic reaction, it seemed possible that a source of energy might aid the reaction and that this energy was supplied by the oxidation-reduction of fumarate and pyruvate. In the present experiments, ATP was chosen as a ready source of chemical energy, and, when added to the reaction mixture, proved to have a great stimulatory effect on the exchange of  $C^{14}O_2$  during decarboxylation of oxalacetate.

Table I shows the effect of ATP upon the exchange, as carried out by crude and partially purified liver preparations. In these experiments, the

decarboxylation was stopped when about 50 per cent complete by the addition of 4 ml. of 10 N  $H_2SO_4$ .

The fractions, labeled "rinse,"  $\beta$ -COOH of oxalacetate, and  $Ce(SO_4)_2$ , were collected as described previously. Under the conditions of this experiment, *i.e.* boiling for 15 minutes with an excess of  $Ce(SO_4)_2$ , pyruvate yields about 1.2 mm of  $CO_2$  per mm of pyruvate present. Presumably, this fraction contains all of the carboxyl group. The various fractions in Table I are listed as mm recovered during the appropriate treatment. In the case of the  $\beta$ -COOH fraction, this value will not indicate the entire amount of oxalacetate remaining at the end of the reaction period, since oxalacetate breaks down spontaneously during the centrifugation and other handling processes previous to collection of the  $\beta$ -COOH fraction. In Tables II, IV, and V, oxalacetate was determined by immediate analysis of an aliquot at the end of the reaction and represents the actual residual oxalacetate. The mm recovered in each fraction are listed as well as the mm of  $C^{13}$  fixed in the total fraction. The latter value was obtained by multiplying the per cent excess  $C^{13}$  in the sample by the mm of carbon in the sample.

In Experiments 1-a and 1-b with purified enzyme, the effect of ATP upon the fixation is striking, 0.04 per cent excess  $C^{13}$  without ATP and 0.22 per cent when ATP was included. Furthermore, an appreciable excess of  $C^{13}$  was found only in the  $\beta$ -COOH fraction. The small excess (0.02 per cent) found in the  $Ce(SO_4)_2$  fraction of Experiment 1-b is of doubtful significance, since the limit of accuracy of the experiment approaches this value and also because there may be a slight contamination of this fraction by oxalacetate not completely removed by the previous treatment. Since the excess  $C^{13}$  is apparently confined to the  $\beta$ -COOH, it is possible to make the following statements: (a)  $C^{13}$  was not fixed by a reaction involving a symmetrical compound or a substance in equilibrium with such a compound. This fact eliminates the possibility of the participation of fumarate (or malate in equilibrium with fumarate) in the initial fixation reaction. (b) The hypothetical shifting of hydroxyl and hydrogen in oxalacetate (Reaction 2),



as proposed by Meyerhof (28), does not occur in the liver preparation. Krampitz *et al.* (4) have already demonstrated that such a reaction does not occur non-enzymatically or with the enzyme preparation obtained from bacteria. (c) Fixation of  $CO_2$  by a linkage with a 2-carbon compound, as was demonstrated with the bacterial preparation (12, 13), does not take place under the conditions of these experiments. This fact is evident since no appreciable amount of  $C^{13}$  was found in the  $Ce(SO_4)_2$  fraction which contains the pyruvate carboxyl.

The effect of ATP is more striking when the enzyme preparations have been dialyzed, but the effect is often noted with untreated (crude) preparations, as is shown by Experiments 2-a and 2-b of Table I. The stimulation caused by ATP with this particular crude extract was almost as great as with the purified enzyme in the previous experiments. As in Experiment 1, an excess of  $C^{13}$  was noted only in the  $\beta$ -COOH fraction.

With another sample of crude extract (Experiments 3-a and 3-b), considerable exchange occurred, even in the absence of added ATP, although addition of ATP caused a further increase. Examination of the  $Ce(SO_4)_2$  fraction of Experiment 3 reveals that considerable  $C^{13}$  was found in this fraction also. Since this fraction was larger than the  $\beta$ -COOH fraction, the mm of  $C^{13}$  recovered were almost equal in the two fractions. The fixation in the  $\alpha$ -COOH of oxalacetate or the  $\gamma$ -COOH of pyruvate may occur in these crude preparations because a symmetrical compound such as fumarate is present as an endogenous substrate and is in equilibrium with the oxalacetate. It is possible that, in extracts in which fixation occurs without added ATP, the ATP or a substitute is supplied by an endogenous reaction.

The  $C^{13}$  content of the "rinse" fractions is typical of the values obtained by this procedure and testifies to the removal of  $C^{13}O_2$  from the solution prior to collection of other fractions. "Rinse" values will be included only in Table III of the subsequent tables since the values never exceeded 0.03 per cent and were rarely that high.

The extent of the exchange in these experiments was somewhat less than that observed in experiments with fumarate and pyruvate by Wood *et al.* (10), but the duration of the experiment and the enzyme concentration were less favorable in the present experiments. It is probable that fixation by Reaction 1 occurs at a rate sufficient to account for the values observed with fumarate and pyruvate. The exchange was somewhat more rapid than that found by Krampitz *et al.* (4) with bacteria, but ATP was not added in their experiments.

A few preparations failed to fix  $C^{13}O_2$  at satisfactory rates in crude or purified form. These preparations, however, usually carried out the decarboxylation of oxalacetate at a normal rate. Hence, the rate of decarboxylation cannot be used as a criterion of ability to perform the reverse or carboxylation reaction. This point will be dealt with more fully in a later section.

*Specificity of Reaction under Consideration*—The method used to obtain the  $\beta$ -COOH group of oxalacetate in the experiments of Table I, *i.e.* boiling at an acid reaction, is not entirely specific for oxalacetate. The possibility must be considered that the compound yielding  $C^{13}O_2$  upon boiling is not oxalacetate, but some other compound which accumulates during the reaction.

Acetoacetate and oxalosuccinate are compounds which fall in this category, since they are very easily decarboxylated. Oxalosuccinate has recently been synthesized by Ochoa (11) and has been shown to be formed from  $\alpha$ -ketoglutarate by fixation of  $\text{CO}_2$  or by oxidation of isocitrate.

The possibility that either of these compounds is involved in the present studies has been investigated in the three ways listed below. (1) Acetoacetate has been eliminated as the source of the fixed  $\text{C}^{13}$  by use of a method which gives decarboxylation of oxalacetate but not of acetoacetate. (2) Oxalosuccinate has been shown to be absent from the reaction mixture by demonstration that  $\alpha$ -ketoglutarate is not a product of decarboxylation by the acid-heat treatment. (Oxalosuccinate yields  $\alpha$ -ketoglutarate on

TABLE II

*Comparison of  $\text{C}^{13}$  Values on Carbon Obtained by Different Methods of Decarboxylation*

Experiment No.	Extract*	Oxalacetate		$\beta$ -COOH of oxalacetate			
		Initial	Final†	Excess $\text{C}^{13}$		Recovered‡	
				Acid-heat	Aniline citrate, 5°	Acid-heat	Aniline citrate, 5°
		mm	mm	per cent	per cent	mm	mm
1	Undialyzed (4 ml.)	1.25	0.40	0.39	0.37		
2	Dialyzed 3.5 hrs. against $\text{H}_2\text{O}$ (6 ml.)	0.80	0.32	0.32	0.37	0.12	0.11

\* Other additions, 0.028 mm of ATP, 0.1 mm of  $\text{MnCl}_2$ , 1.3 mm of  $\text{NaHC}^{13}\text{O}_3$  ( $\text{C}^{13} = 4.69$  per cent excess), 1 mm of Na pyruvate in a total volume of 21 ml.; time, 45 minutes.

† Amount found by respective methods in one-half the final mixture. Part of the final oxalacetate breaks down spontaneously during the time of preparation for the degradation.

‡ Determined on an aliquot at the end of the incubation period.

decarboxylation.) (3) The sum of the residual oxalacetate and pyruvate is shown to be equal to the original amount of oxalacetate. It is thus apparent that there has been no removal of oxalacetate or pyruvate by side reactions.

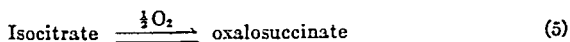
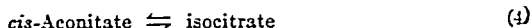
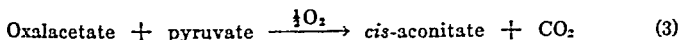
*Acetoacetate*—In the experiments of Table II twice the usual volume of reactants was employed, and the reaction was stopped by addition of 10 ml. of 50 per cent citric acid. After removal of the  $\text{C}^{13}\text{O}_2$  in the usual way, the solution was divided into two equal parts, and separate rinses were obtained. The decarboxylation was carried out in two ways, method (a) by boiling at an acid reaction, as in the experiments of Table I, and method (b) by the use of aniline citrate at 5°. Greville (25) has shown that the decarboxylation of acetoacetate by aniline citrate is negligible at 5° and



that oxalacetate can be determined in the presence of acetoacetate by carrying out the reaction at a low temperature. Therefore, if acetoacetate is present, the results given by the two methods should vary, since method (a) will yield  $\text{CO}_2$  from both acetoacetate and oxalacetate while method (b) will decarboxylate only the latter compound. Any  $\text{C}^{13}$  found by method (b) will necessarily be formed from a compound other than acetoacetate.

An examination of the results of these experiments shows that the  $\text{C}^{13}$  content of fractions obtained by the two methods checks reasonably well with both dialyzed and undialyzed preparations. Also, in the one experiment in which the amount of  $\text{CO}_2$  in the fractions was obtained quantitatively by the two methods, the amount by method (b) was as large as that obtained by method (a). These results eliminate acetoacetate as a factor in the reaction under study.

*Oxalosuccinate*—It seemed possible that this substance was formed from the oxalacetate and that, in part at least, the  $\text{C}^{13}$  was fixed in the oxalosuccinate by Ochoa's reaction. According to current concepts, the formation of oxalosuccinate from oxalacetate and pyruvate involves the following steps:



Preliminary experiments demonstrated that the liver preparation contains the oxalosuccinate carboxylase<sup>1</sup> and that therefore there was a possibility of such a fixation with this preparation. In order to decide this question, the oxalosuccinate was determined in the experiment described in Table III. This experiment was carried out in the usual way with an incubation of 45 minutes. The determination of oxalosuccinate was accomplished by decarboxylation of the oxalosuccinate to  $\alpha$ -ketoglutarate, oxidation of the  $\alpha$ -ketoglutarate to succinate, and determination of the succinate by the succinioxidase method (23). For the determination, 97 per cent of the total of solution involved in the 45 minute experiment of Table III was used. After acid-heat treatment the material was subjected to continuous ether extraction for 24 hours. The extract was oxidized with  $\text{KMnO}_4$  (29) and then reextracted with ether. The ether-soluble material, which contained the succinate, was taken up in water, neutralized, and was then tested with the succinioxidase preparation. The entire extract

<sup>1</sup> We wish to express our appreciation to Dr. Severo Ochoa for his gift of oxalosuccinate.

was used in a single manometer vessel containing 3.0 ml. of reaction fluid. No succinate was detected, although by this method as little as 1 mg. of succinate should be detected, which on the basis of molarity would be about one-seventieth of the original or one-twentieth of the final oxalacetate. Appropriate controls were run with the succinoxidase preparation to establish its activity and, after failure to detect succinate in the sample, a known sample of succinate was added to the experimental material to insure that no inhibitor was present. The added succinate was readily oxidized. These results show that oxalosuccinate is not formed in sig-

TABLE III

*Changes in Total Pyruvate (Oxalacetate Plus Pyruvate) during Fixation Reaction*

<i>C<sup>13</sup></i> determinations				
	$\beta$ -COOH of oxalacetate		Rinse	
	Recovered	Excess <i>C<sup>13</sup></i>	Recovered	Excess <i>C<sup>13</sup></i>
	<i>mM</i>	<i>per cent</i>	<i>mM</i>	<i>per cent</i>
30 sec. incubation .....	0.53	0.05	1.48	0.03
45 min. " .....	0.20	0.24	1.52	0.03
Pyruvate determinations				
	Specific method (b)	Non-specific method (a)	Ratio, 420:540 readings	
	<i>mM</i>	<i>mM</i>		
30 sec. incubation .....	0.64	0.66	1.28	
45 min. " .....	0.64	0.67	1.29	

Each experiment contained 3.5 ml. of pigeon liver extract (dialyzed 3 hours against 0.9 per cent KCl); 0.014 mM of ATP; 0.05 mM of MnCl<sub>2</sub>; 0.65 mM of oxalacetate (in 0.65 mM of Na<sub>2</sub>PO<sub>4</sub>); 0.72 mM of NaHC<sup>13</sup>O<sub>3</sub> (*C<sup>13</sup>* = 3.65 per cent excess) in a total volume of 10.0 ml.; incubated for indicated length of time.

nificant amounts under the conditions of these experiments and that it is not the source of the fixed carbon. In addition, the experiment indicates that neither  $\alpha$ -ketoglutarate nor succinate is formed in the reaction.

*Oxalacetate and Pyruvate*—If the conversion is correctly expressed by Reaction 1, formation of pyruvate at the expense of the oxalacetate will constitute the only change occurring during the experiment. Then, if the residual oxalacetate is converted to pyruvate at the end of the experiment by acid-heat treatment, the amount of pyruvate should be the same in a fermented as in a control (non-fermented) sample. On the other hand, if other reactions such as those described in Reactions 3 to 7 are involved, a

net decrease in pyruvate should be noted, since the equivalent of 2 molecules of pyruvate is used in the formation of the tricarboxylic acid molecule.<sup>2</sup>

The colorimetric method of Friedemann and Haugen (22) was used for the determination of the pyruvate. This method was also used as an additional check on the formation of oxalosuccinate, since it can be adapted to detect  $\alpha$ -ketoglutarate in the presence of pyruvate. Procedure (a) of the method serves this purpose and involves the use of ethyl acetate as a solvent and a reaction period of 25 minutes. Under these conditions the method is relatively non-specific and is applicable to  $\alpha$ -ketoglutarate, oxalacetate, and presumably other  $\alpha$ -keto acids, as well as pyruvate. The light absorption of the alkaline hydrazones is measured at different wave-lengths and the ratio of these two absorptions is calculated. The variation of the ratio from that given by pyruvate indicates the presence of other keto acids such as  $\alpha$ -ketoglutarate. Procedure (b) of Friedemann and Haugen's method involves the use of toluene or xylene as a solvent and a reaction period of 5 minutes. Under these conditions, the method is relatively specific for pyruvate.

In the experiments described in Table III these two procedures were used and, in addition, the C<sup>13</sup> fixed was determined by the usual procedure. In the control experiment, the reaction was stopped after about 30 seconds by washing the contents of the manometer flask into a volumetric flask containing 10 ml. of 10 per cent HPO<sub>3</sub>. After dilution to 50 ml, the solution was centrifuged and the proteins were washed once with 10 ml. of 1 per cent HPO<sub>3</sub>. The wash liquid was added to the original centrifugate and the combined solutions were treated in the usual way to obtain the "rinse" sample and  $\beta$ -COOH sample. The boiling procedure used in collection of the latter sample converted any remaining oxalacetate to pyruvate, or correspondingly the oxalosuccinate to  $\alpha$ -ketoglutarate. The pyruvate was then determined in aliquots by procedures (a) and (b) of the Friedemann and Haugen method. The experiment with a 45 minute incubation period was treated in the same way.

<sup>2</sup> Examination of the columns labeled " $\text{Ce}(\text{SO}_4)_2$  fraction" of Tables I and V shows that in every case this fraction was smaller when ATP was present than in the control experiment. This finding might be interpreted as an indication that either pyruvate or oxalacetate had been removed by side reactions. However, in other experiments not presented here, the variations in the  $\text{Ce}(\text{SO}_4)_2$  fraction are in the opposite direction. These variations are probably due to inequalities in treatment of different samples since the yield of 1.2 mm of CO<sub>2</sub> per mm of pyruvate varies according to the heating period. In experiments in which a rigorous attempt was made to control the heating period, no appreciable differences were noted between experiments incubated with and without ATP. Furthermore, this equality is confirmed by the more quantitative colorimetric procedure of Friedemann and Haugen (22).

With regard to the  $C^{13}$  values, the "rinse" values for both the control and 45 minute experiments were reasonably low (Table III). A small excess of  $C^{13}$  (0.05 per cent) was found in the  $\beta$ -COOH of the oxalacetate in the control experiment. Since this figure is higher than the "rinse" value, it appears that the oxalacetate contained a small amount of  $C^{13}$  even after an incubation period of only 30 seconds. After 45 minutes, the excess was much higher, 0.24 per cent indicating that substantial fixation had occurred. The bicarbonate used in this experiment contained only 3.63 per cent excess  $C^{13}$ , which was somewhat lower than that employed in previous experiments.

Neither the specific nor non-specific method of pyruvate determination (Table III) indicated that any decrease in total pyruvate (oxalacetate plus pyruvate) had occurred in conjunction with the  $CO_2$  fixation process. Furthermore, the non-specific method gave a negligible increase over the specific method, pointing to the probable absence of  $\alpha$ -keto acids other than pyruvate.

In the last line of Table III, the ratios of the light absorption of the alkaline hydrazones for Filters 420 and 540 of the Klett-Summerson instrument are calculated. Pyruvate standards at three levels gave an average value of 1.30 for this ratio, but the replacement of 10 per cent of the pyruvate by an equivalent amount of  $\alpha$ -ketoglutarate raised the ratio to 1.42 and 25 per cent  $\alpha$ -ketoglutarate to 1.57. The ratios in the control and 45 minute experiments were 1.28 and 1.29 respectively, giving no evidence of the presence of  $\alpha$ -ketoglutarate, and accordingly of oxalosuccinate previous to the decarboxylation.

All of the above results are thus in complete accord with the idea that the only conversion which occurs is that shown in Reaction 1. Furthermore, since pyruvate was recovered in an amount equivalent to the original oxalacetate, it is clear that the fixed  $C^{13}O_2$  was obtained by cleavage of a compound which yielded pyruvate or a compound analyzed as pyruvate. This observation gives substantial evidence that the compound which contained the fixed carbon was oxalacetate or a compound with very similar properties.

*Influence of Pyruvate,  $Mg^{++}$ , and  $Mn^{++}$  upon Fixation Reaction*—Pyruvate and  $MnCl_2$  were added to the reaction mixture in most of the previous experiments.  $MnCl_2$  has previously been reported by Evans *et al.* (9) to be essential for the fixation of isotopic  $CO_2$  in the fumarate-pyruvate system. Krampitz *et al.* (4) also found that either  $Mn^{++}$  or  $Mg^{++}$  was a component of the bacterial system. Pyruvate was included in the previous experiments because it was thought that a high concentration of this substance might aid the fixation reaction, since it is apparently closely related to one of the components. The necessity of these substances has been investigated and the results are presented in Table IV. Experiment 1 demonstrated that the complete system gave an excellent exchange (0.52

per cent excess). The omission of pyruvate (Experiment 2) gave a slightly higher result, showing that pyruvate was not a necessary component. Presumably, pyruvate was formed with sufficient rapidity by the enzymatic and spontaneous decarboxylation of oxalacetate to fulfil any requirements there might be for pyruvate.

Omission of  $MnCl_2$  (Experiment 3) had a very deleterious effect on the exchange reaction, giving a final excess of 0.06 per cent in the  $\beta$ -COOH. There was also a somewhat smaller decarboxylation (0.175 mM, as compared to 0.237 mM) in this experiment, although the decrease by no means parallels the decrease in  $C^{13}$  fixation. Experiment 4 demonstrated that  $MgCl_2$  cannot replace  $MnCl_2$  in the fixation reaction. The results of the latter two experiments agree with the findings of Evans *et al.* (9), who re-

TABLE IV

*Effect of  $MnCl_2$ ,  $MgCl_2$ , and Na Pyruvate upon Fixation of  $C^{13}$  in Oxalacetate*

Experiment No.	Description	$\beta$ -COOH of oxalacetate		Ce(SO <sub>4</sub> ) <sub>2</sub> fraction	
		Final*	Excess $C^{13}$	Recovered	Excess $C^{13}$
		mM	per cent	mM	per cent
1	Complete	0.175	0.52	1.04	0.00
2	Pyruvate omitted	0.173	0.57	0.60	0.02
3	$MnCl_2$ omitted	0.237	0.06	1.01	0.02
4	" replaced by $MgCl_2$	0.199	0.11	0.96	0.01

\* Oxalacetate, as determined on an aliquot at the end of the incubation period. The complete mixture (Experiment 1) contained 3.5 ml. of liver extract (dialyzed against KCl), 0.014 mM of ATP, 0.65 mM of  $NaH^{13}CO_3$  (excess  $C^{13}$  = 4.41 per cent), 0.5 mM of oxalacetate in 0.5 mM of  $Na_2PO_4$ , 0.5 mM of Na pyruvate, and 0.05 mM of  $MnCl_2$  in a total volume of 12.5 ml.; time, 45 minutes.

ported that  $Mn^{++}$  could not be replaced by  $Mg^{++}$  in the decarboxylation of oxalacetate to pyruvate by pigeon liver preparations. Ochoa and Weisz-Tabori (11) in their recent experiments on the decarboxylation of oxalosuccinate reported a similar  $Mn^{++}$  specificity. From these results it is clear that  $Mn^{++}$  is an essential component of the system for the fixation reaction. It should be noted that inorganic phosphate has been added in all experiments, although it is not known whether or not it is necessary for the fixation process.

In no case did a significant excess of  $C^{13}$  appear in the Ce(SO<sub>4</sub>)<sub>2</sub> fraction.

*Substitution of Adenylic Acid for Adenosine Triphosphate*—If ATP is serving only as a phosphate carrier in the fixation reaction, it should be possible to replace ATP by adenylic acid (AA). On the other hand, if the function of ATP is related to its ability to serve as a phosphate donor or as a source of energy, substitution of AA should not be successful.

In Table V, two series of three experiments each are reported, repre-

senting (1) a control experiment, (2) the addition of ATP, and (3) a similar experiment in which AA has been added instead of ATP. If AA can successfully replace ATP, excess  $C^{13}$  in the  $\beta$ -COOH of oxalacetate should be approximately equal in the latter two experiments.

In Series A, the control experiment showed a fixation of 0.03 per cent in the  $\beta$ -COOH, but the addition of ATP increased the value to 0.31 per cent. Substitution of AA gave a figure of 0.10. Although this by no means equals the values of the ATP experiment, it represents a tripling over the control experiment. As was pointed out in a previous section, the AA contains a small amount of ATP or ADP (2 per cent calculated as ATP),

TABLE V

*Effect of Substitution of Adenylic Acid (AA) for Adenosine Triphosphate (ATP) in Fixation Reaction*

Series	Description	$\beta$ -COOH of oxalacetate		Ce(SO <sub>4</sub> ) <sub>2</sub> fraction	
		Final*	Excess $C^{13}$	Recovered	Excess $C^{13}$
		<i>mM</i>	<i>per cent</i>	<i>mM</i>	<i>per cent</i>
A	1. Control	0.23	0.03	1.35	0.04
	2. ATP added	0.19	0.31	1.02	0.01
	3. AA "	0.17	0.10	1.29	0.02
B	1. Control	0.22	0.03	0.97	
	2. ATP added	0.22	0.16	0.87	
	3. AA "	0.20	0.03	0.95	0.01

\* Determined on aliquot at the end of the reaction period.

Series A, each experiment contained in a total volume of 12.5 ml., 3.5 ml. of dialyzed liver extract; 0.6 mM of oxalacetate dissolved in 0.6 mM of Na<sub>2</sub>PO<sub>4</sub>; 0.65 mM of NaHC<sup>13</sup>O<sub>3</sub> (excess  $C^{13}$  = 4.4 per cent); 0.05 mM of MnCl<sub>2</sub>; 0.5 mM of Na pyruvate and if indicated; 0.014 mM of ATP or AA; incubated for 45 minutes. Series B, the same as Series A except that 3.0 ml. of dialyzed extract and 0.65 mM of NaHC<sup>13</sup>O<sub>3</sub> (excess  $C^{13}$  = 5.0 per cent) were used.

and it is possible that this small amount of ATP has some effect on the reaction. However, in Series B with another sample of liver extract, the addition of AA gave no increase over the control value, although the addition of ATP gave a 5-fold increase.

From these two series of experiments, it must be concluded that AA replaces ATP very poorly, or not at all. It remained possible, however, that the AA was not active because of its destruction by enzymes in the liver. This possibility is in line with Kalckar's suggestion (30) that the failure of AA to replace ATP in certain systems may be due to the destruction of AA by agencies which do not attack ATP readily. Kalckar cited the failure of AA to replace ATP in Ochoa's experiments with  $\alpha$ -ketoglutarate oxidation (31) as a possible example.

The inactivation of AA by the liver system, therefore, has been investigated (Table VI). The test was made by determining the ability of added AA to act as a phosphate acceptor before and after a 45 minute incubation with the liver enzymes. It is realized that the possibility exists that the criterion chosen for detection of inactivation in these experiments is not infallible. For example, it is possible that the deamination product of AA, inosinic acid, may act as a P acceptor, but on the other hand may not function in the fixation reaction.

In conducting the test, phosphoglycerate was added as a source of phosphopyruvate and the latter was allowed to phosphorylate AA. The

TABLE VI

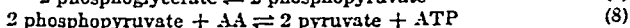
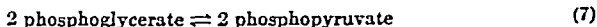
*Effect of Incubation with Liver Extract on Adenylic Acid As a Phosphate Acceptor for Phosphoglyceric Acid*

Experiment No	Adenylic acid	Preliminary incubation*	Changes in P (all values corrected for original controls)		
			Inorganic P	Adenosine triphosphate	Phosphopyruvate
	mm		mg $\times 10^{-3}$	mg $\times 10^{-3}$	mg $\times 10^{-3}$
1			0.75	0.31	10.17
2	$7 \times 10^{-4}$		5.94	4.48	7.51
3		45 min	1.18	0.12	12.61
4	$7 \times 10^{-4}$	45 "	4.86	4.22	7.86

\* Liver extract incubated with adenylic acid at 38° at pH 7.4 prior to the addition of phosphoglyceric acid. In addition to adenylic acid, the flasks contained 0.3 ml of dialyzed liver extract;  $7.5 \times 10^{-3}$  mm of Na phosphoglycerate,  $5 \times 10^{-3}$  mm of  $\text{MnCl}_2$ ,  $2 \times 10^{-3}$  mm of inorganic phosphate,  $4.8 \times 10^{-3}$  mm of  $\text{NaHCO}_3$  in a total volume of 1.05 ml. The time of incubation of the extract with phosphoglycerate was 45 minutes.

changes in inorganic, 9 minute acid-hydrolyzable P and alkaline-iodine P fractions were measured. The ATP fraction (9 minute acid-hydrolyzable) was corrected for phosphopyruvate (alkaline-iodine-labile) upon the basis that one-half of the latter is hydrolyzed in 9 minutes in 1 N acid at 100°.

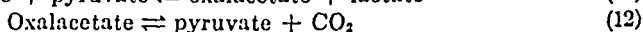
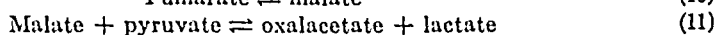
In Experiments 1 and 2, no preliminary incubation period was employed. If no AA was added (Experiment 1), little change occurred in the inorganic and ATP fractions, although a large amount of phosphopyruvate was formed. The inclusion of AA (Experiment 2) caused a much larger increase in both the inorganic and ATP fractions, partially at the expense of phosphopyruvate. The reactions involved are



It is thus clearly demonstrated that AA is active in the enzyme system, as employed. In Experiments 3 and 4, a preliminary incubation period of 45 minutes, under conditions comparable to those used in the fixation experiments, preceded the addition of the phosphoglycerate. During this period, AA was in contact with the liver enzymes. At the end of 45 minutes, phosphoglycerate was added and an additional incubation period of 45 minutes similar to the procedure in Experiments 1 and 2 was followed. Examination of Experiments 3 and 4 shows that results roughly parallel to those of Experiments 1 and 2 were obtained and that there is little evidence that the ability of AA to serve as a P acceptor was impaired by the preliminary incubation. Theoretically, the AA added in these experiments should be able to accept  $4.34 \times 10^{-2}$  mg. of P, if converted to ATP. In both cases, the increase in ATP approaches this figure, indicating complete phosphorylation. If any of the AA had been inactivated by the previous incubation, the total ATP formed should have been decreased by an equivalent amount.

There is no evidence, then, that the failure of AA to replace ATP was due to a loss of its ability to serve as a P acceptor during contact with the liver preparation.

*Effect of Adenosine Triphosphate on Fixation of CO<sub>2</sub> in Fumarate-Pyruvate System*—As mentioned earlier, Evans *et al.* (9) and Wood *et al.* (10) studied CO<sub>2</sub> fixation with the same type of enzyme preparation but used fumarate and pyruvate as substrates. These studies were performed with dialyzed enzymes in the absence of added ATP. However, "crude" diphosphopyridine nucleotide (DPN) was included in the reaction mixture. Presumably the reactions proceed as follows:



Through this series of reversible reactions, C<sup>13</sup>O<sub>2</sub> becomes incorporated in the carboxyl groups of all of the compounds taking part in the reaction (10). Since ATP appeared to exert a decided effect on the fixation of CO<sub>2</sub> when oxalacetate was the only substrate, it appeared of interest to determine whether ATP had an effect on the fumarate-pyruvate system. Such a study is described in Table VII. With fumarate as the starting point the fixation reaction depends upon (a) the accumulation of oxalacetate through Reactions 10 and 11 and (b) the rate of the exchange reaction itself (Reaction 12). The amount of oxalacetate formed by Reaction 11 is equivalent to the lactate formed (second column, Table VII). The lactate, therefore, is a rough indication of the extent of Reaction 11.

The amount of C<sup>13</sup> incorporated was determined by oxidizing the reaction



mixture with excess  $\text{Ce}(\text{SO}_4)_2$  at  $100^\circ$  for 15 minutes. This method produces about 1.2 mm of  $\text{CO}_2$  per mm of pyruvate or lactate and about 2.8 mm of  $\text{CO}_2$  per mm of malate. Presumably, all of the carboxyl groups of the above compounds are liberated by this procedure. Fumarate yields an inappreciable amount of  $\text{CO}_2$  under the same conditions. The determination gives a measure of the  $\text{C}^{13}$  in the carboxyl groups of lactate, pyruvate, and malate with about 25 per cent dilution by carbon from other positions, but the  $\text{C}^{13}$  in the carboxyl groups of fumarate will not be recovered. Since, according to Krebs *et al.* (32), the ratio of fumarate to malate under these conditions is about 1:3, a large part of the  $\text{C}^{13}$  will be recovered. The last column represents a calculation of the mm of  $\text{C}^{13}$  fixed.

TABLE VII

*Effect of Adenosine Triphosphate (ATP), Adenylic Acid (AA), and Diphosphopyridine Nucleotide (DPN) on  $\text{C}^{13}\text{O}_2$  Fixation with Fumarate and Pyruvate As Substrate*

Experiment No.	Additions	Lactic acid formed	$\text{Ce}(\text{SO}_4)_2$ fraction		
			Recovered	Excess $\text{C}^{13}$	$\text{C}^{13}$ fixed
		mm	mm	per cent	mm $\times 10^{-4}$
1		0.036	1.20	0.03	3.2
2	DPN 0.005 mm	0.063	1.08	0.08	9.0
3	ATP 0.014 "	0.065	1.07	0.12	13.1
4	DPN 0.005 " + ATP 0.014 mm	0.094	0.99	0.23	22.6
5	" 0.005 " + AA 0.014 mm	0.074	1.10	0.11	11.9

\* Other components, 3.5 ml. of liver extract (dialyzed against KCl), 0.35 mm of Na fumarate, 0.35 mm of Na pyruvate, 0.05 mm of  $\text{MnCl}_2$ , 0.4 mm of phosphate buffer (pH 6.2), 0.65 mm of  $\text{NaHC}^{13}\text{O}_2$ ; excess  $\text{C}^{13}$  = 4.41 per cent) in a total volume of 12.0 ml.; time, 60 minutes.

It should be noted that analyses by the aniline citrate method of several preliminary experiments showed that no appreciable amount of oxalacetate accumulated during the reaction. Therefore, the acid-heat treatment was omitted in these experiments.

If neither ATP nor DPN was added (Experiment 1, Table VII), only 0.036 mm of lactate was formed, and little  $\text{C}^{13}$  ( $3.2 \times 10^{-4}$  mm) was fixed. The addition of DPN almost doubled the lactate formed and gave a  $\text{C}^{13}$  fixation of  $9 \times 10^{-4}$  mm. ATP alone gave about the same amount of lactate but raised the  $\text{C}^{13}$  fixed to  $13.1 \times 10^{-4}$  mm. These effects of ATP and DPN (Experiment 4) appeared to be additive as far as both the lactate production and  $\text{C}^{13}$  fixation were concerned. From these experiments, it appears that ATP has a stimulatory effect upon Reaction 11 (lactate formation) as well as upon the fixation reaction. The stimulatory effect of

ATP upon malic dehydrogenase also has been studied by the Thunberg method of methylene blue reduction. In these experiments also, ATP caused a distinct stimulation (unpublished experiments).

In Experiment 5, an attempt was made to substitute AA for ATP. DPN was also present in this experiment. The lactate production (0.074 mM) was slightly higher than with DPN alone (Experiment 2), and the  $C^{14}$  fixation was similarly increased. However, neither value was high enough to suggest that AA could replace ATP in the system.

From these preliminary experiments, it can be concluded that ATP exerts an effect on  $CO_2$  fixation in the fumarate-pyruvate system, and that this effect is partially due to a stimulation of the formation of oxalacetate from malate. It is somewhat puzzling to note that Evans *et al.* (9) as well as Wood *et al.* (10) obtained excellent fixation with about the same system without added ATP. The explanation for this partial discrepancy is not apparent, although it is possible that even higher fixation would have been obtained if ATP had been included. In one experiment, Evans *et al.* (9) reported that  $Mn^{++}$  and DPN did not entirely replace the stimulatory effect of boiled tissue extract, suggesting that some other factor was involved. It is interesting to note that Schlenk and Schlenk (33) reported that impure DPN (75 per cent) contained up to 15 per cent adenosine-5-monophosphate (AA) or ATP as an impurity. The method used did not distinguish between the two compounds. In all of the experiments with the fumarate-pyruvate system, impure DPN has been added and it is possible that considerable ATP was added in this way.

#### DISCUSSION

At the present time, the nature of the stimulatory effect of ATP upon the fixation of  $CO_2$  in oxalacetate is not clear. Two possible modes of action are suggested: (a) stimulation through phosphorylation of a cofactor of the reaction and (b) stimulation via direct participation of ATP in the reaction.

To illustrate the first possibility, the function of ATP might be to phosphorylate thiamine to diphosphothiamine, with the latter compound playing a part in the fixation reaction. Diphosphothiamine has been suggested by Smythe (34) to have such a rôle, although Krampitz *et al.* (5) could find no support for such a claim. In any case, the phosphorylation of thiamine by ATP has been demonstrated (35-37). As a second example of phosphorylation of a cofactor by ATP, Gunsalus *et al.* (38) believe that pyridoxal is phosphorylated by ATP and that the resulting compound is a coenzyme of tyrosine decarboxylase. Other similar reactions are conceivable. The possibility that ATP acts in the present reaction by phosphorylating some coenzyme is weakened somewhat by the fact that activation by ATP occurs even after purification of the preparation by  $(NH_4)_2SO_4$  precipitation and

dialysis. These procedures usually remove readily dissociable coenzymes, and it would be expected that the ATP reaction would be lost after purification. However, it should be noted that the purification procedures were not sufficiently exhaustive to remove certain of the tightly bound coenzymes (*cf.* for example Umbreit *et al.* (39) and Green *et al.* (40)).

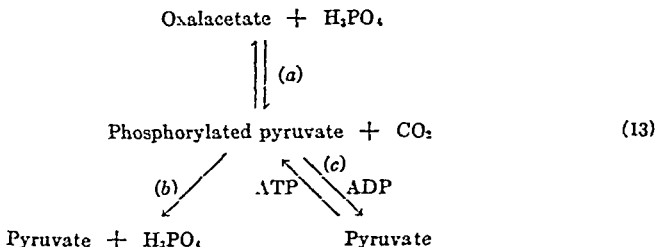
The second possibility, direct participation of ATP in the fixation reaction, could come about through the phosphorylation of one or more of the compounds involved. Possible intermediates include phosphopyruvate and labile phosphate derivatives of pyruvate, oxalacetate, and  $\text{CO}_2$ .

Work now in progress on the rôle of ATP indicates that diphosphothiamine and phosphopyruvate will not replace ATP in the fixation reaction, virtually eliminating these two possibilities from the foregoing lists.

As mentioned previously, there is not a perfect correlation between the ability of a preparation to fix  $\text{CO}_2$  in oxalacetate and its ability to decarboxylate oxalacetate. In liver preparations, the amount of  $\text{C}^{13}$  fixed in the  $\beta\text{-COOH}$  has varied from 0.03 per cent excess to 0.65 per cent excess, even though the conditions of the experiments were similar and ATP was added in both cases. In spite of the marked difference in the rate of fixation, the rates of decarboxylation of oxalacetate differed only slightly.

This situation obtains also when the enzyme preparations are obtained from rat and chicken liver. An extract of an acetone powder of chicken liver fixed a significant amount of  $\text{C}^{13}$  in oxalacetate but the values did not approach those obtained with pigeon liver. Nevertheless, the rate of decarboxylation of oxalacetate was comparable to that of pigeon liver. Extracts of lyophilized homogenates of rat liver decarboxylate oxalacetate quite rapidly but may exhibit very poor fixation of  $\text{C}^{13}\text{O}_2$ . The above facts strongly suggest that the decarboxylation and fixation reactions are not identical in all phases and that a single enzyme does not mediate both processes.

As a possible explanation, Reaction 13 is presented. In this series of



reactions, the fixation reaction is assumed to occur by the junction of  $\text{CO}_2$  with a phosphorylated derivative of pyruvate (a). This derivative is not

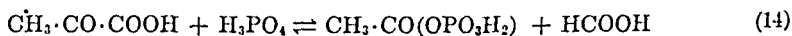
necessarily the well known enol-phosphate compound. As a further assumption, the intermediate compound can undergo two additional reactions: (b) an irreversible phosphatase reaction resulting in the formation of pyruvate and  $\text{H}_2\text{PO}_4$  and (c) a reversible reaction with the ATP-ADP system. In such a system, fixation of  $\text{CO}_2$  by (a) will probably be dependent in part upon the maintenance of a suitable concentration of the phosphorylated intermediate. In turn, the concentration of this substance will depend upon a balance of all the reactions and in particular of (b) and (c). If the synthesis of phosphorylated pyruvate from pyruvate and ATP (c) is sufficiently rapid in comparison with (b), a suitable concentration of the intermediate will be maintained and fixation can occur. Furthermore, since the synthesis via (c) will depend on the presence of the necessary enzyme and the concentration of pyruvate and ATP, the stimulatory effect of ATP can be explained.

On the other hand, the poor correlation between fixation and decarboxylation of oxalacetate can be accounted for by another type of balance in the same series of reactions. If (b) of Reaction 13 is sufficiently rapid with respect to (c) and to (a), little fixation would occur, although decarboxylation would be rapid.

It is perhaps worth while to point out that the methods employed in the present study necessitate the halting of the reaction before equilibrium is reached, since it is desired that a suitable quantity of oxalacetate be left for analysis. Therefore, the important consideration of any series of reactions such as those described is one of comparative rates rather than one of final equilibrium point.

It should be emphasized that the above series of reactions is illustrative only and is not intended as a proposal of the actual mechanism of  $\text{CO}_2$  fixation. It can be shown that any scheme involving a minimum of two paths for either the decarboxylation reaction or a subsequent reaction will yield a similar result; *i.e.*, a non-correlation between the fixation and decarboxylation reactions.

Although the foregoing example is hypothetical, it is possible to point to an analogous situation in the reversible reaction involving pyruvate, acetyl phosphate, and formate (Reaction 14) (12, 13):



In this system acetyl phosphate can undergo two further reactions, (a) an irreversible dephosphorylation to acetate and inorganic phosphate and (b) a reversible reaction with the ATP system. It has been found (41) that high concentrations of inorganic phosphate inhibit the phosphatase reaction (a). When the concentration of phosphate was increased in studies with  $\text{C}^{13}$  formate, it was shown that acetyl phosphate accumulated and

also that the reaction of acetyl phosphate with formate to yield  $C^{13}$  pyruvate was likewise enhanced (13).

#### SUMMARY

1. The fixation of  $C^{13}O_2$  in the  $\beta$ -COOH of oxalacetate has been demonstrated with a preparation obtained from pigeon liver. Little or no  $C^{13}O_2$  is fixed in the  $\alpha$ -COOH of oxalacetate, indicating that no symmetrical compounds are involved. The fixation is of sufficient speed to account for the total fixation of  $CO_2$  previously reported for this type of preparation.

2. The fixation of  $CO_2$  in oxalacetate is greatly aided by the presence of adenosine triphosphate, especially in dialyzed preparations. Adenylic acid will not replace adenosine triphosphate in the system.

3. Evidence is presented that the conversion of oxalacetate to pyruvate and  $CO_2$  represents the over-all reaction under study. In particular, the possible participation of oxalosuccinate and acetoacetic acid is eliminated.

4.  $Mn^{++}$  is necessary for the fixation reaction and  $Mg^{++}$  will not serve as a replacement.

5. Adenosine triphosphate also stimulates the fixation of  $C^{13}O_2$  when fumarate and pyruvate are used as substrates. Adenosine triphosphate appears to stimulate the formation of oxalacetate from fumarate as well as the incorporation of  $C^{13}$  in the former compound.

6. A possible explanation for the observed lack of correlation between decarboxylation as compared to carboxylation is presented.

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# IDENTIFICATION OF THE LINOLEIC AND LINOLENIC ACIDS OF BEEF TALLOW

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(Received for publication, March 8, 1946)

Hilditch and Longenecker (1), in an investigation of the component acids of beef tallow, failed to obtain the tetrabromostearic acid, m.p. 115°, characteristic of vegetable oil linoleic acid (*cis,cis*-9,12-linoleic acid), upon bromination of the fraction which contained the octadecadienoic acid. In addition, upon oxidation of this fraction with potassium permanganate, they obtained only small yields of tetrahydroxystearic acids, whereas yields of at least 40 per cent may be expected from the linoleic acid of vegetable oil (2). Failure to obtain the usual identifying tests for *cis,cis*-9,12-linoleic acid was in agreement with results obtained by other workers who studied the octadecadienoic acids obtained from numerous other fats of animal origin (3). The octadecadienoic acid of lard, however, gives fair yields of the tetrabromostearic acid and the tetrahydroxystearic acids characteristic of the linoleic acid of vegetable oil (3). Since the octadecadienoic acid of beef tallow had been shown by oxidative splitting to be a 9,12-linoleic acid, it was concluded by these earlier workers that it must be a geometrical isomer of vegetable oil linoleic acid.

It seemed surprising to us that there should be a difference between the octadecadienoic acids of beef tallow and lard, and it was considered desirable to reinvestigate the nature of the octadecadienoic acid of beef tallow, and, at the same time, to attempt to characterize the trienoic acid which spectrophotometric analysis had shown to be a usual, although minor, constituent of this fat (4).

## EXPERIMENTAL

The best grade of edible beef tallow (fatty acid composition shown in Table I), obtained from the internal organs of the animal, was employed. Its fatty acid composition was calculated from spectrophotometric analysis (4) and the iodine number (48.3). The tallow was converted to fatty acids by the rapid, large scale, laboratory saponification procedure previously reported and the saturated acids and the oleic acid were removed by crystallization from acetone at -20° and -60°, respectively (5). The filtrate acids from the -60° crystallization contained more than 90 per

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cent of the polyunsaturated acids originally present in the tallow. From 20,000 gm. of tallow, 2000 gm. of these filtrate acids (iodine number 105.2, neutralization equivalent 290) were obtained. This material was then further fractionated as shown in the accompanying diagram. The apparatus and technique employed in the low temperature crystallizations have been reported previously (6). It should be pointed out that throughout the entire fractionation procedure distillation was employed in only one step (F-2 to D-2), and this was a rapid, straight run, vacuum distillation at 1 mm. through an 18 inch Vigreux column, which has a very low pressure drop. Distillation was conducted in this manner to avoid overheating and, perhaps, isomerizing the polyunsaturated acids.

Fractions F-3, P-4, and F-4 were analyzed spectrophotometrically (4) for polyunsaturated acids; their composition is shown in Table II.

TABLE I

*Fatty Acid Composition of Edible Beef Tallow Employed As Starting Material*

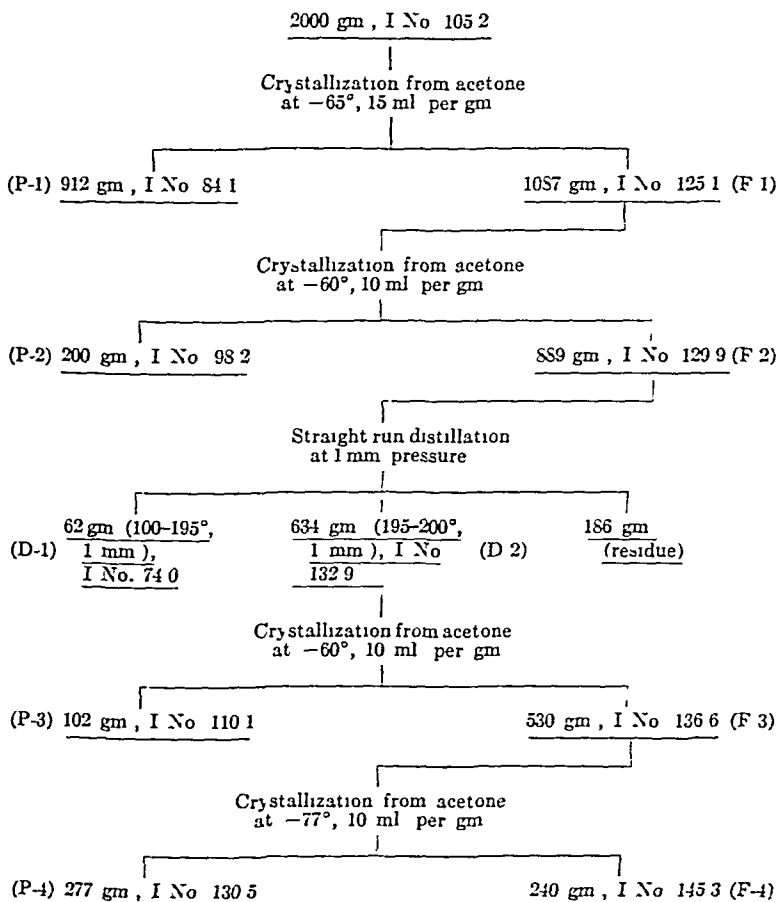
	<i>per cent</i>
Oleic acid.....	46.3
Saturated acids.....	50.3
Non-conjugated diene acids.....	2.2
"        triene " .....	0.46
"        tetraene acids.....	0.09
Conjugated diene acids.....	0.66
"        triene " .....	0.025
"        tetraene acids.....	0.002

*Bromination and Isolation of Solid Bromides*—To a well stirred solution of 95 gm. of Fraction P-4 (Table II and diagram) dissolved in 2000 ml. of petroleum ether (boiling range, 30–75°) and cooled to –5° to –10°, 86 gm. of bromine were added dropwise over a 20 minute period, during which the temperature rose to about –3°. A white solid precipitated during the addition of bromine. The mixture was stirred for  $\frac{1}{2}$  hour at –5° after the bromine addition was complete. Amylene was added until the bromine color had disappeared; the solution was kept at –20° overnight, after which it was filtered by suction. After the precipitate was washed with 500 ml. of petroleum ether at –20°, it was a white, crystalline solid and weighed 33.9 gm. Its melting point was 109–115°, a small quantity remaining unmelted to 133°. This was finely ground and washed several times with boiling ethyl ether to separate it into an ether-soluble (30.8 gm.) and an ether-insoluble portion (3.1 gm.). The ether-insoluble portion, m.p. 171–174°, was crystallized twice from xylene, yielding 1.8 gm. of 9,10,12,13,15,16-hexabromostearic acid, m.p. 180°. When it was



mixed with the hexabromostearic acid prepared from perilla oil fatty acids, the melting point was unchanged.

*Fractionation of Polyunsaturated Acid Fraction Obtained  
from 20,000 Gm of Edible Beef Tallow\**



\* P = precipitate, F = filtrate, D = distillate

The ether-soluble portion was crystallized at -20° from a mixture of ethyl ether and petroleum ether, yielding 241 gm. of 9,10,12,13-tetra-bromostearic acid, m p. 112-112.7°. When it was mixed with the tetra-

bromostearic acid obtained from corn oil fatty acids, the melting point was unchanged.

Fraction F-4 (95 gm.) was brominated as described above with 96 gm. of bromine; 38.1 gm. of insoluble bromides, m.p. 123–139°, were obtained. This precipitate was separated into an ether-soluble portion (25.5 gm.) and an ether-insoluble portion (12.6 gm.). The ether-insoluble portion was crystallized twice from xylene (small quantities of bromides insoluble in boiling xylene were discarded), yielding 5.1 gm. of 9,10,12,13,15,16-hexabromostearic acid, m.p. 170–171.5°, with a small amount remaining unmelted at 198°. When it was mixed with the hexabromostearic acid prepared from perilla oil fatty acids, the melting point was unchanged. The small amount remaining unmelted at 198° probably consisted of bromides of acids containing more than three double bonds.

The ether-soluble material from the bromination of Fraction F-4 was crystallized from a solution of ethyl ether and petroleum ether. The yield

TABLE II  
*Spectrophotometric Analysis of Fractions F-3, P-4, and F-4 for Polyunsaturated Acids*

Fraction	Non-conjugated acids			Conjugated acids		
	Diene	Triene	Tetraene	Diene	Triene	Tetraene
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
F-3	24.7	7.5	0.91	4.6	0.13	0.007
P-4	26.3	5.0	0.23	4.4	0.10	0.004
F-4	23.5	9.5	1.8	5.8	0.20	0.008

was 13.2 gm. of 9,10,12,13-tetrabromostearic acid, m.p. 108.5–113°. When the substance was mixed with the tetrabromostearic acid obtained from corn oil fatty acids, the melting point was unchanged.

#### DISCUSSION

On the basis of the spectrophotometric analysis (Table II), it is possible to calculate the yield of solid tetrabromostearic and hexabromostearic acids obtainable from Fractions P-4 and F-4, assuming that only 50 and 25 per cent of the theoretical yields of these acids can be obtained from the non-conjugated diene and triene acids, respectively (7, 8). These yields are compared, in Table III, with those actually obtained.

From the high yields of solid bromides shown in Table III, one must conclude that the non-conjugated octadecadienoic and trienoic acids of beef tallow consist mainly of *cis,cis*-9,12-octadecadienoic and *cis,cis,cis*-9,12,15-octadecatrienoic acids, respectively. Stated another way, the major proportion of the 9,12-linoleic and 9,12,15-linolenic acids of beef

tallow has the same geometrical configuration as the linoleic and linolenic acids of vegetable oil. Our data do not preclude the possibility that beef tallow contains other non-conjugated isomers of linoleic and linolenic acids in small proportions, since our bromide yields were always less than expected. Low yields of solid bromides might be expected, however, even if all the non-conjugated linoleic and linolenic acids present were identical with vegetable oil linoleic and linolenic acids, since the yield of solid bromides is affected markedly by the other component acids of the mixture under investigation and low yields are clearly related to the concentration of oleic and saturated acids, which interfere with the complete precipitation of the solid bromides (9).

Also worthy of note is the fact that the iodine numbers of Fractions F-3, P-4, and F-4 as calculated from the spectrophotometric data are 3.8, 2.6, and 6.6 units lower than the iodine numbers determined. In calculating

TABLE III

*Calculated Yields of 9,10,12,13-Tetrabromostearic and 9,10,12,13,15,16-Hexabromostearic Acids and Yields Obtained from 95 Gm. Each of Fractions P-4 and F-4*

Fraction	9, 10, 12, 13-Tetrabromostearic acid		9, 10, 12, 13, 15, 16-Hexabromostearic acid	
	Calculated	Found	Calculated	Found
	gm.	gm.	gm.	gm.
P-4	26.7	24.1	3.3	1.8
F-4	23.8	13.2	6.3	5.1

the iodine number from the spectrophotometric data, it was assumed that the Wijs solution adds to all the double bonds in the conjugated acids and that no saturated acids were present. The large excess of Wijs solution with respect to conjugated acids makes the first assumption reasonable. The discrepancy is greater still, however, when one considers that saturated material is undoubtedly present in these fractions. The discrepancy is not due to inaccuracy of the methods, since on comparison the iodine number of soy bean and cottonseed oils (per cent of oleic and saturated acids known) calculated from spectrophotometric data usually agrees within 1 unit of the iodine number determined. One possible explanation for the discrepancy is that tallow contains small proportions of polyunsaturated acids, which have been concentrated in Fractions F-3, P-4, and F-4, in which the double bonds are too far apart to be conjugated by means of alkali under the conditions employed in the spectrophotometric analysis. These acids cannot, therefore, be determined spectrophotometrically, but they must react in the normal manner with Wijs iodine solution.

Also of interest is the fact that the ratio of conjugated diene to non-

conjugated diene fatty acids in the starting material and in Fractions F-3, P-4, and F-4 is surprisingly high. This is somewhat unexpected, but it has been confirmed by examining other samples of tallow spectrophotometrically.

Establishment of the fact that the non-conjugated di- and triunsaturated acids of beef tallow consist primarily of *cis,cis*-9,12-linoleic acid and *cis,cis,cis*-9,12,15-linolenic acid, respectively, is of some significance from a nutritional standpoint, since it would indicate that beef tallow (as well as lard) is satisfactory as a source of the so called "essential" fatty acids (the linoleic acid of lard having been shown previously to be *cis,cis*) (3).

The authors are indebted to B. A. Brice and Margaret L. Swain, of this Laboratory, for the spectrophotometric analyses.

#### SUMMARY

Crystalline 9,10,12,13-tetrabromostearic and 9,10,12,13,15,16-hexabromostearic acids, identical with those obtained from vegetable oils, have been isolated in good yields from the polyunsaturated acids of edible beef tallow. It has been concluded, therefore, that the non-conjugated octadecadienoic and trienoic acids of beef tallow consist mainly of *cis,cis*-9,12-linoleic and *cis,cis,cis*-9,12,15-linolenic acids, respectively.

On concentrates of polyunsaturated acids obtained from edible beef tallow, discrepancy between the iodine values determined and those calculated from spectrophotometric data suggests the possibility of the presence in this fat of minor proportions of polyunsaturated acids with double bonds too far apart to be conjugated with alkali under the conditions employed in the spectrophotometric analysis.

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## LETTERS TO THE EDITORS

### BIOLOGICAL ACETYLTATION OF NATURAL AMINO ACIDS\*

Sirs:

In 1910 Knoop suggested the occurrence of N-acetyl derivatives as intermediates in the biological synthesis of amino acids.<sup>1</sup> This hypothesis gained support from the work of du Vigneaud and Irish<sup>2</sup> and from subsequent work of du Vigneaud *et al.*<sup>3</sup> Both acetic acid<sup>4</sup> and pyruvic acid<sup>5</sup> are

*Aerobic Incubation of Rat Liver Slices with Deuterio Acetic Acid (59 Atom Per Cent Excess Deuterium) and Amino Acids (0.01  $\mu$ ) at 37°*

Time of incubation, 3 hours, buffer volume, 50 ml per gm of wet liver.

Amino acid added (A)	Molarity of deuterio acetate in buffer (B)	Atom per cent D in acetyl group of isolated acylamino acid (C)	Mg acylamino acid formed per gm. wet liver <sup>a</sup> (D)
<i>L</i> -Leucine	0.03	1.1	0.36
"	0.04	1.00 (1.00)†	0.51
<i>L</i> -Phenylalanine	0.03	1.20 (1.20)†	0.41
"	0.04	1.27 (1.22)†	0.44
<i>L</i> -Phenylaminobutyric acid	0.02	9.2	2.5

\* Calculated from the equation  $x = (C/(C_0 - C))y$ , where  $C$  = atom per cent D in the acetyl group,  $C_0$  = atom per cent D in the acetic acid added,  $y$  = mg. of normal acetyl amino acid added

† Analysis after second recrystallization.

sources of acetyl in the acetylation of phenylaminobutyric acid in intact animals. Since it is unlikely that the acetyl derivatives of natural amino acids would accumulate *in vivo* in quantities sufficient for isolation, the acetylation has been investigated in an *in vitro* system.

\* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and from the Nutrition Foundation, Inc

<sup>1</sup> Knoop, F, *Z. physiol. Chem.*, **67**, 489 (1910)

<sup>2</sup> du Vigneaud, V, and Irish, O. J., *J. Biol. Chem.*, **122**, 349 (1937-38).

<sup>3</sup> du Vigneaud, V., Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **131**, 273 (1939)

<sup>4</sup> Bernhard, K., *Z. physiol. Chem.*, **267**, 91 (1940)

<sup>5</sup> Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944), **159**, 45 (1945).

Rat liver slices were incubated in Krebs' phosphate buffer to which had been added deuterio acetic acid and either *l*-leucine, *l*-phenylalanine, or *l*-phenylaminobutyric acid. After incubation, small quantities of the corresponding non-isotopic acetylamino acids were added, as carriers, to the supernatant buffer fluid and isolated after ether extraction and recrystallization from water of the ether-soluble residue. The presence of deuterium<sup>6</sup> in the isolated compounds demonstrates that acetylation of the amino acids had occurred (see the table). Non-enzymatic exchange was eliminated by a control experiment without tissue. Since breakdown of acetylamino acids under the same experimental conditions has been observed, it is likely that the quantities of acetylamino acids formed during the entire experimental period were considerably greater than is indicated from the calculated values. Moreover, acetylphenylaminobutyric acid which, once formed, is not further metabolized, accumulates in much larger quantities than the acetyl derivatives of the two natural amino acids.

The observed acetylation of leucine and phenylalanine in a biological system suggests strongly that acetylamino acids are products in the normal intermediary metabolism of amino acids and that acetylation is not confined to amines and amino acids which are foreign to the animal body.

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<sup>6</sup> Micro deuterium analyses were carried out by an unpublished method developed by D. Rittenberg.

# THE ANTAGONISM OF SULFONAMIDES BY PTEROYL- GLUTAMIC ACID AND RELATED COMPOUNDS

Sirs:

We have been studying the relation between pteronic acid derivatives<sup>1</sup> (*L. casei* factor, folic acid, etc.) and sulfonamides in various bacteria. The interactions of the sulfonamides and folic acid in animal metabolism have been reviewed recently.<sup>2</sup> It had also been noted that the inhibition of *Lactobacillus arabinosus* by sulfapyridine was reversed by a factor in liver extract which resembled folic acid.<sup>3</sup>

We have noted that organisms requiring the preformed derivatives for

*Growth and Antisulfonamide Activity for Streptococcus faecalis Strains\**

Compound	<i>S. faecalis</i> (Ralston)					<i>S. faecalis</i> R, half maximum growth
	Half maximum antagonism of sulfadiazine (SD)				50 per cent increase in turbidity	
	1 $\gamma$ SD per ml	10 $\gamma$ SD per ml	100 $\gamma$ SD per ml.	1000 $\gamma$ SD per ml.		
<i>p</i> -Aminobenzoic acid	0.003	0.03	0.3	2.0	0.001	Inactive
<i>p</i> -Aminobenzoylglu- tamic acid..	1	30	300	>300	0.05	"
Pteroylglutamic acid†	0.0003	0.0003		0.0003	0.0001	0.00015
Pteroyltriglutamic acid†.	0.004	0.004	0.004	0.003	0.003	0.003
Thymine	0.06	0.25	0.25	0.25	0.25	0.3-0.5

\* All values are given in micrograms per ml., after 24 hours incubation.

† The pteroylglutamic acid is a sample of synthetic material; the pteroyltriglutamic acid a preparation isolated from a fermentation liquor. Both were generously furnished by Dr. B. L. Hutchings and Dr. E. L. R. Stokstad of the Lederle Laboratories, Pearl River, New York.

growth are relatively insensitive to sulfonamides. On the usual basal media for lactobacilli (free of *p*-aminobenzoic acid) the growth of *L. casei* and of *Streptococcus faecalis* R on pteroylglutamic acid or on thymine is not inhibited at 16 hours by 1280  $\gamma$  of sulfadiazine per ml.

The related organism *S. faecalis* (Ralston) grows without added pteroyl-

<sup>1</sup> Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 103, 667 (1946).

<sup>2</sup> Daft, F. S., and Sebrell, W. H., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*, New York, 3, 49 (1945).

<sup>3</sup> Tepley, L. J., Axelrod, A. E., and Elvehjem, C. A., *J. Pharmacol. and Exp. Therap.*, 77, 207 (1943).

glutamic acid, but is stimulated by this and related compounds. The data of the table show that a competitive inhibition occurs between sulfadiazine and *p*-aminobenzoic acid or *p*-aminobenzoyl-*l*-glutamic acid.<sup>4</sup> The antagonism by the pteric acid derivatives or by thymine is non-competitive; i.e., a relatively constant amount is required to antagonize any level of the sulfonamide. These amounts approximate those required to stimulate the growth of this strain in the absence of sulfonamides or to support growth of *S. faecalis* R. The synthesis of pteroylglutamic acid, etc., from *p*-aminobenzoic acid must then be the point of sulfonamide inhibition under these conditions. Similar results have been obtained with sulfanilamide, sulfathiazole, and sulfapyridine or by use of *L. arabinosus* 17-5 and a series of enterococci.

It has not been possible to demonstrate a similar non-competitive antagonism with *Escherichia coli*, *Staphylococcus aureus*, or *Diplococcus pneumoniae*. This indicates that pteroylglutamic acid probably does not interfere with sulfonamide therapy with most organisms.

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<sup>4</sup> Auhagen, E., *Z. physiol. Chem.*, 277, 197 (1943).



## PROTEIN-PHYTIC ACID RELATIONSHIP IN PEANUTS AND COTTONSEED

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Phytin, the calcium, magnesium, and potassium salt of phytic acid (inositol hexaphosphoric acid), is the principal storage form of phosphorus and inositol in all seeds (1-5). Although phytin or phytic acid was long considered to occur only in plants, recent work has shown that its occurrence is more widespread. Thus Rapoport and Guest (6) have demonstrated that the erythrocytes of the chicken and turtle contain appreciable amounts of phytic acid, while other investigators (7-10) have shown that phytin is also present in various soils. Furthermore, the nutritional significance of inositol (11) and phytic acid (12-14) is fairly well established. Phytic acid exhibits rachitogenic properties because it is capable of forming insoluble salts with calcium that are not absorbed through the intestinal wall. This rachitogenic activity can be destroyed under certain conditions by the enzyme phytase (15) which normally occurs in most seeds.

In the course of investigations on peanut and cottonseed meals in this Laboratory, it was found that there is present in these meals a dialyzable substance that exerts a pronounced effect on the solubility characteristics of the meal proteins (16). This dialyzable meal constituent has now been identified as phytin. Since phytin is normally present in seed meals in relatively large quantities, and since its effectiveness in influencing the solubility characteristics of the meal proteins is marked, it was deemed advisable to investigate thoroughly the protein-phytic acid relationship in seeds with the view of contributing toward the eventual elucidation of the rôle played by phytic acid in the economy of the plant and of pointing the way to possibilities for the fuller industrial utilization of the protein constituents of both peanut and cottonseed meals. The protein-phytic acid solubility relationship over a wide pH range has been determined for peanut and cottonseed meals and the corresponding dialyzed meals and isolated proteins, and is presented in this paper. A limited number of comparative data on the protein-phytic acid solubility relationship in

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soy bean meal are also included. Since the naturally occurring enzyme phytase rapidly hydrolyzes phytic acid to inositol and phosphoric acid, a few of the characteristics of the phytase present in the seed meals investigated are also presented.

### *Materials and Methods*

*Peanut Meal*—A composite sample of a Pearl variety of peanuts (white skins) (17), grown in 1943, was used in this work. The peanuts were mechanically shelled, cracked, and flaked in the early summer of 1944, and the oil was removed from the flakes by extraction with a continuous flow of cold Skellysolve F (a naphtha solvent, b.p. 30–60°). Residual solvent was removed by aeration at room temperature, and the extracted flakes were ground to produce a fine meal. The meal<sup>1</sup> contained 0.762 per cent total phosphorus and 0.070 per cent inorganic phosphorus (9.18 per cent of the total), as thus prepared.

*Cottonseed Meal*—Fresh cottonseed meats received from a Mississippi mill in 1943 were cracked and flaked, and the oil was extracted in a large Soxhlet type extractor by means of ethyl ether. Residual solvent was removed by aeration at room temperature, and hull particles were removed from the oil-free meats by means of a Raymond separator mill. The meal<sup>1</sup> so obtained contained 1.532 per cent total phosphorus and 0.080 per cent inorganic phosphorus (5.21 per cent of the total).

*Soy Bean Meal*—A small batch of an unidentified variety of soy beans (1943 crop) was ground in a meat chopper and extracted in a glass Soxhlet apparatus with Skellysolve F. After the initial extraction, the excess solvent was removed at room temperature, and the partially defatted

<sup>1</sup> Analysis of several batches of solvent-extracted meals prepared from seeds obtained at different times has shown some variation in the amounts of total and inorganic phosphorus present. These variations can possibly be explained by a difference in the maturity of the seeds, as has been pointed out by Earley and DeTurk for corn (5). Through the kind cooperation of Dr. J. W. Neely, Stoneville, Mississippi, a series of immature cottonseed bolls was obtained for analysis. The bolls were picked at intervals of 5, 15, 21, and 30 days after flowering. They were opened, and the lint was removed from the seeds by hand, after which the seeds were dried at 105°, ground, and extracted with Skellysolve F. The meals contained, respectively, on a moisture- and oil-free basis, 1.05, 0.48, 0.49, and 1.02 per cent total phosphorus and 0.263, 0.17, 0.201, and 0.214 per cent inorganic phosphorus (25, 35.4, 41.0, and 20.9 per cent of the total). When it is considered that the oil-free meal from mature cotton seeds contains approximately 1.5 per cent total phosphorus and approximately 0.08 per cent (5 per cent of the total) inorganic phosphorus, it is apparent that in the period between 21 days after flowering and maturity of the cotton seed there is an increase in the content of organic phosphorus with a concomitant decrease in the content of inorganic phosphorus. It is logical to assume that similar changes may occur during maturation in all seeds.

seeds were again ground and extracted with Skellysolve F. After evaporation of the solvent at room temperature, the meal was sieved to remove a large percentage of the hulls before being ground. The meal<sup>1</sup> contained 0.755 per cent total phosphorus and 0.034 per cent inorganic phosphorus (4.6 per cent of the total).

*Peanut Protein*—Peanut meal was extracted with a dilute sodium hydroxide solution (pH 8.0), and the extract was clarified by centrifugation. The protein was precipitated at pH 4.5 by the addition of hydrochloric acid, and recovered by centrifugation. The moist protein was suspended in water (thymol was added), transferred to cellophane (Visking) membranes, and dialyzed against running distilled water at 4° for 3 days. The suspension was then dried by lyophilization (18). The dried protein had the following composition: moisture 10.4 per cent, nitrogen 15.43 per cent, ash 0.31 per cent, total phosphorus 0.645 per cent, and inorganic phosphorus 0.014 per cent.

*Cottonseed Protein*—Ethyl ether-extracted cottonseed meal was extracted by being stirred occasionally with sodium hydroxide solution (pH 11.0) for  $\frac{1}{2}$  hour, and the extract was removed by centrifugation. The protein was precipitated at pH 4.0 by the addition of hydrochloric acid, and was then treated exactly as described above for the preparation from peanuts. The dried protein had the following composition: moisture 10.64 per cent, nitrogen 14.0 per cent, ash 0.22 per cent, total phosphorus 1.164 per cent, and inorganic phosphorus 0.013 per cent.

*Nitrogen Determination Procedures*—(a) The procedure used for determining the percentage of total meal nitrogen peptized has been given in detail previously (19) but was, briefly, as follows: 2.5 gm. portions of the meal, contained in separate 200 ml. screw cap centrifuge bottles, were treated with 100 ml. of water containing sufficient sodium hydroxide solution or acid solution to give the final pH value desired. The suspensions were allowed to stand for 3 hours at room temperature with occasional shaking and were then clarified in the centrifuge. The pH values of the extracts were determined by means of a glass electrode, and total nitrogen was determined on duplicate aliquots by either the semimicro- or the macro-Kjeldahl method. All of the nitrogen values reported in this paper were calculated on the basis of the total volume of solvent added in each case.

(b) The procedure used for determining the percentage of total nitrogen peptized for dialyzed meal samples was as follows: 2.5 gm. portions of the meals were transferred to cellophane membranes with 50 ml. of water and dialyzed at 4° against running distilled water for 48 hours. The contents of the membranes were transferred to 100 ml. glass-stoppered graduated cylinders and the volumes were adjusted to 100 ml. by adding water and sufficient sodium hydroxide solution or acid solution to give the final pH

values desired. The remainder of the procedure was exactly as described under (a), except that, in calculating the percentage of total nitrogen peptized, the total nitrogen of the dialyzed meal was considered to be equal to the total nitrogen of the original meal minus that lost during dialysis.

(c) The procedure employed for determining the percentage of total nitrogen peptized in experiments in which the isolated protein was used was exactly as given under (a) above, except that only 1.25 gm. of protein were treated with 100 ml. of solvent.

*Total Phosphorus*—Usually, the phosphorus analyses were carried out on aliquots of the same solutions used for determining nitrogen solubility. Total phosphorus was determined either by the method of Fontaine (20), with the spectrophotometer at a wave-length of 820 m $\mu$ , or by a modification of the reduced molybdate method of Gerritz (21) with the Evelyn colorimeter with a No. 660 filter.

*Inorganic Phosphorus*—Extraction of inorganic phosphorus was accomplished by a modification of the procedure of Berenblum and Chain (22). The method may be described briefly as follows: Equal volumes of the meal extracts and 12 per cent trichloroacetic acid (usually 5 ml. of each) were pipetted into 15 ml. conical centrifuge tubes, mixed, and centrifuged. 2 ml. of the clear supernatant solution were pipetted into a 125 ml. separatory funnel marked at 20 ml.; then 1.5 ml. of 10 N H<sub>2</sub>SO<sub>4</sub> and 5 ml. of 5 per cent ammonium molybdate were added, and the mixture was diluted to 20 ml. with distilled water and mixed by swirling. 10 ml. of isobutyl alcohol were added, after which the funnel was shaken for 1½ minutes and the aqueous layer was discarded. The isobutyl alcohol layer was washed with two successive 10 ml. portions of approximately 1.0 N H<sub>2</sub>SO<sub>4</sub>, and the washings were discarded. Then 15 ml. of the stock solution of SnCl<sub>2</sub> (10 gm. of SnCl<sub>2</sub>·6H<sub>2</sub>O in 25 ml. of concentrated HCl), diluted (1:200) with approximately 1.0 N H<sub>2</sub>SO<sub>4</sub>, were added to the isobutyl alcohol layer, and the mixture was shaken for 30 seconds. The aqueous layer was discarded. The blue isobutyl alcohol layer was transferred to a 50 ml. volumetric flask and diluted to volume with 95 per cent ethyl alcohol.

The intensity of the blue color was measured 1 hour after development in an Evelyn photoelectric colorimeter equipped with a No. 660 filter. The intensity of the blue color developed was found to become stable approximately 40 minutes after development and to remain stable for at least 19 hours.

When meal and protein samples were analyzed directly, inorganic phosphorus was extracted by shaking a 1 gm. sample of the material with 50 ml. of 6 per cent trichloroacetic acid for 1 hour. The mixture was filtered through paper, and a 2 ml. aliquot of the filtrate was used for analysis, as

outlined above. A more detailed investigation of the inorganic phosphorus method used in the work reported here has been published by Pons and Guthrie (23).

#### EXPERIMENTAL

*Solubility of Nitrogen and Phosphorus<sup>2</sup> Compounds of Peanut, Cottonseed, and Soy Bean Meals in Hydrochloric Acid and Sodium Hydroxide Solutions over Wide pH Range*—The solubility of the nitrogen and phosphorus compounds in peanut meal extracts, as a function of pH, is shown in Fig.

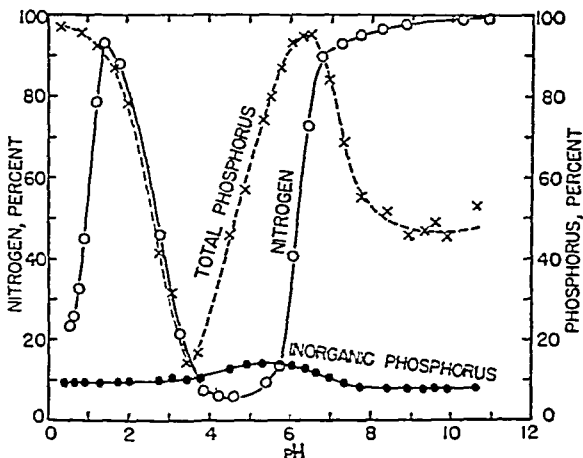


FIG. 1. The percentage of the total nitrogen and phosphorus of solvent-extracted peanut meal which is soluble in hydrochloric acid and sodium hydroxide solutions at different pH values. The inorganic phosphorus values are reported as percentage of the total meal phosphorus.

1. It is at once apparent that the solubility of the phosphorus compounds does not correspond to that of the nitrogenous constituents. A very sharp minimum (15 per cent) in the total phosphorus solubility curve occurs at pH 3.5, whereas the minimum solubility range for the nitrogenous constituents extends from pH 3.8 to 5.5. Total phosphorus solubility increases very rapidly on either side of the minimum to maximum values (about 97 per cent) at pH 0.5 and 6.5. It is evident, however, that between pH 3.5 and 1.5 the total phosphorus solubility is dependent upon the nitrogen

<sup>2</sup> Since practically all of the phosphorus of the oil-free seed meals is present as phytin, and since practically all of the nitrogen is present as protein, the results can be considered as representative of the solubilities of phytic acid and protein.

solubility, indicating that most of the phosphorus (85 per cent) apparently is combined with the proteins at pH 3.5. This conclusion appears to be justified, since the proteins at pH 3.5 have a net positive charge and should be capable, therefore, of reacting with phytic acid. From pH 3.5 to 6.5 the total phosphorus solubility increases more rapidly than does the nitrogen solubility, indicating that as the proteins pass through their isoelectric points the protein-phytic acid complexes dissociate. For example, at pH 5.8 only 13 per cent of the meal nitrogen is in solution, whereas 86.6 per cent of the phosphorus is soluble.

At pH values lower than 1.5 the solubility of the meal proteins decreases rapidly, while there is a slight increase in the percentage of the total phosphorus which is soluble. The present evidence leads to the belief that this effect arises from the complete displacement of the phytic acid ion by the chloride ion at pH values lower than 1.5. At pH values higher than 6.5 the opposite result is obtained; namely, the percentage solubility of total phosphorus decreases, while that of the nitrogen increases. In this instance precipitation of the normally soluble phytate ion results from the formation of an insoluble mixed magnesium, calcium, and potassium salt of phytic acid.

The percentage of the total phosphorus of the peanut meal found to be inorganic phosphorus over the entire pH range is also shown in Fig. 1. From pH 0.4 to 1.7 (1.0 N to 0.05 N HCl) a constant value (9.7 per cent) was found for the inorganic phosphorus content of these extracts. At pH values above 1.7 there was a significant increase in the amount of inorganic phosphorus present in the extracts. This increase is undoubtedly due to the enzymatic activity of the phytase<sup>3</sup> of the peanut meal, since the time elapsing between the preparation of the extract and analysis usually was 4 hours. A maximum solubility value of 14.2 per cent inorganic phosphorus was obtained at pH values of 4.93, 5.32, and 5.52 (0.0075 N, 0.005 N, and 0.004 N HCl, respectively), followed by decreasing solubility until a constant value of 7.9 per cent was obtained between pH 8.91 and 10.61 (0.006 N and 0.015 N NaOH, respectively). These results indicate that the pH of optimum activity for the peanut phytase is in the range pH 4.8 to 6.0 at 25°. In this connection, it should be pointed out that the minimum solubility value for inorganic phosphorus in the pH range 8.91 to

<sup>3</sup> Germination of peanut and cottonseed at 30° between moist filter paper (in the dark) showed a rapid conversion of organic phosphorus to inorganic phosphorus. After 0, 24, 48, 72, and 96 hours germination, the seeds were dried at 105°, and the oil was extracted with Skellysolve F. The percentage of the total phosphorus present as inorganic phosphorus after the times indicated was 12.2, 11.2, 15.7, 23.2, and 32.4, respectively, for the peanut, and 10.0, 11.2, 24.9, 35.4, and 44.4, respectively, for the cottonseed.

10.61 is 2 per cent lower than that in the pH range 0.4 to 1.7. Upon autolysis for 24 hours at 25°, meal extracts having pH values within the range 0.4 to 1.7 or 8.91 to 10.61 showed no appreciable increase in inorganic phosphorus. However, a very rapid increase did occur in the inorganic phosphorus content as the result of autolysis of extracts having pH values within the range of optimum phytase activity (pH 4.8 to 6.0).

The solubility of the nitrogen and phosphorus compounds in cottonseed meal extracts, as a function of pH, is shown in Fig. 2. With cottonseed meal extracts, as with peanut meal extracts, the solubility of the phosphorus compounds does not correspond to that of the nitrogenous constituents. However, the solubility of total phosphorus obtained at various pH values for cottonseed meal differs from that obtained for peanut meal. With cottonseed meal a minimum total phosphorus solubility (17 per cent) is observed at approximately pH 2.75. Increasing amounts of phosphorus become soluble on either side of this pH value. Maximum phosphorus solubility (85 per cent) occurs at pH 0.70 and 6.0. However, at neither of these pH values does the nitrogen solubility curve approach that for total phosphorus. The cottonseed phosphorus solubility curve does approach the nitrogen solubility curve between pH 2.75 and 1.5, and it may be assumed that the association between protein and phytic acid is greatest in this pH range.

The percentage of the total meal phosphorus which is soluble between pH 7.0 and 11.0 is much less for cottonseed than for peanut. This behavior may be explained on the assumption that the ratio of magnesium and calcium to potassium in the cottonseed exceeds that in the peanut; hence, there is a greater probability that a more insoluble mixed salt of phytic acid will be formed in alkaline extracts of cottonseed meal.

The results of analyses for inorganic phosphorus in cottonseed meal extracts covering the entire pH range are also shown in Fig. 2. A minimum value of 4.8 per cent-soluble inorganic phosphorus was found in the pH range 0.32 to 1.98 (1.0 N to 0.05 N HCl, respectively). A maximum value of 7.9 per cent-soluble inorganic phosphorus was obtained at pH values of 4.0, 4.2, and 4.4 (0.02 N, 0.0175 N, and 0.015 N HCl, respectively), followed by decreasing solubility, until a value of 2.5 per cent was obtained between pH 8.65 and 11.08. Thus the optimum pH range for cottonseed phytase<sup>3</sup> activity lies between pH 4.0 and 5.0 at 25°, as contrasted to a range of pH 4.8 to 6.0 found for peanut phytase.

The solubility of the nitrogen and phosphorus compounds in soy bean meal extracts, as a function of pH, is shown in Fig. 3. The nitrogen solubility curve for soy bean meal is quite similar to that shown for peanut meal (Fig. 1), except at the lower pH values where nitrogen solubility in the case of soy bean meal exceeds that for peanut meal. A minimum (15

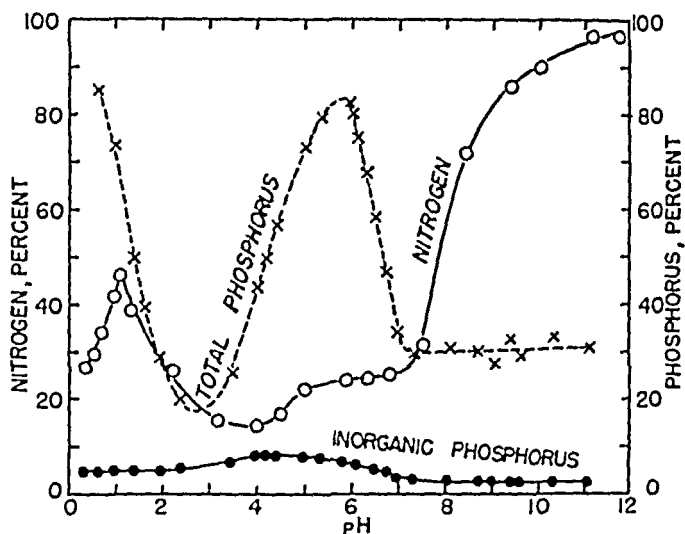


FIG. 2. The percentage of the total nitrogen and phosphorus of solvent-extracted cottonseed meal which is soluble in hydrochloric acid and sodium hydroxide solutions at different pH values. The inorganic phosphorus values are reported as percentage of the total meal phosphorus.

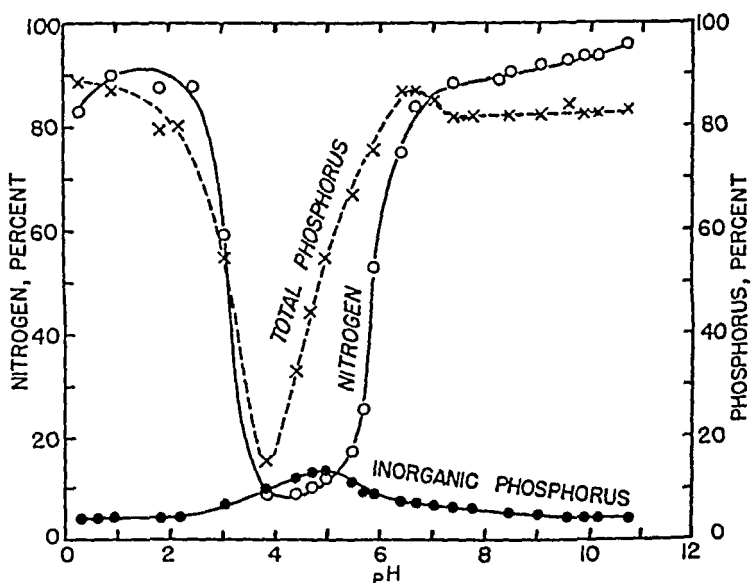


FIG. 3. The percentage of the total nitrogen and phosphorus of solvent-extracted soy bean meal which is soluble in hydrochloric acid and sodium hydroxide solutions at different pH values. The inorganic phosphorus values are reported as percentage of the total meal phosphorus.



per cent) in the total phosphorus solubility curve is obtained at pH 3.85 and the maxima (around 87 per cent) at pH values of 0.28 and 6.4 to 6.6. Of particular interest is that part of the soy bean phosphorus solubility curve which lies above pH 7.0 where, in contrast to peanut and cottonseed, there is very little decrease in the solubility of phosphorus compounds. It is evident, then, that, although the soy bean and peanut meals used in these experiments contain practically the same amount of phosphorus, an alkaline extract (above pH 8.0) of soy bean meal will contain almost twice as much soluble phytin phosphorus as will a similar extract prepared from peanut meal. The practical importance of this fact will be discussed in a later section.

The percentage of the total soy bean meal phosphorus, which was found to be inorganic phosphorus in extracts of different pH values, is also shown in Fig. 3. A minimum inorganic phosphorus solubility value of 4.6 per cent was found in the pH range 0.28 to 1.82 (1.0  $N$  to 0.05  $N$  HCl, respectively) and a maximum of 13.5 per cent in the pH range 4.7 to 4.98 (0.01  $N$  to 0.0075  $N$  HCl, respectively), followed by a decrease to a minimum value of 4.6 per cent in the pH range 9.5 to 10.7 (0.0075  $N$  to 0.015  $N$  NaOH, respectively). The minimum inorganic phosphorus solubility values in acid and alkaline solutions are the same in the case of the soy bean, whereas the minima differ slightly in the case of both the peanut and cottonseed (Figs. 1 and 2).

*Comparison of Solubility of Nitrogen and Phosphorus Compounds of Peanut and Cottonseed in Hydrochloric, Sulfuric, and Trichloroacetic Acids*—The solubilities of the nitrogen and phosphorus compounds in peanut meal, as a function of pH and of the various acids used to adjust the pH, are shown in Fig. 4, and the corresponding results for cottonseed meal are shown in Fig. 5. A very sharp minimum in phosphorus solubility occurs at pH 3.5 in the case of peanut meal (Fig. 4) when hydrochloric acid is used as the extractant, whereas a rather broad minimum solubility range (pH 2.0 to 4.0) results when sulfuric and trichloroacetic acids are used. These differences in the broadness of the minimum phosphorus solubility zone, when these three acids are used to extract peanut meal, can be attributed to the fact that sulfuric and trichloroacetic acids differ appreciably from hydrochloric acid in their ability to peptize peanut meal proteins in the pH range 3.5 to 1.5. In the pH range 3.5 to 6.5 the amount of phosphorus which is soluble is independent of the particular acid employed in adjusting the pH of the peanut meal suspension. This behavior provides further evidence of the dissociation of protein-phytic acid complexes at pH values higher than 3.5, as has been brought out previously.

The phosphorus solubility curves for cottonseed meal (Fig. 5) approximate each other over the entire pH range investigated, regardless of the

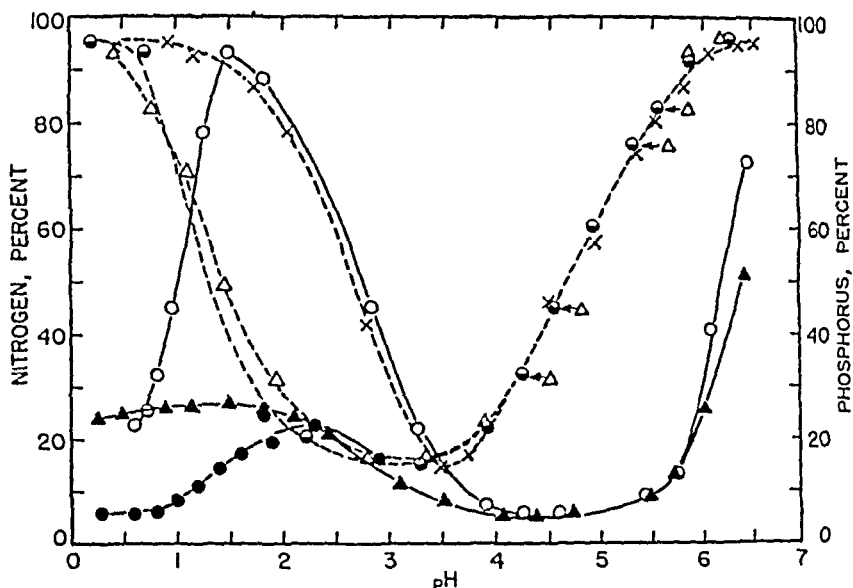


FIG. 4. The percentage of the total nitrogen and phosphorus of solvent-extracted peanut meal, which is soluble in hydrochloric, sulfuric, and trichloroacetic acid solutions at different pH values. HCl,  $\circ$  nitrogen,  $\times$  phosphorus;  $\text{H}_2\text{SO}_4$ ,  $\blacktriangle$  nitrogen,  $\triangle$  phosphorus;  $\text{CCl}_3\text{COOH}$ ,  $\bullet$  nitrogen,  $\bullet$  phosphorus.

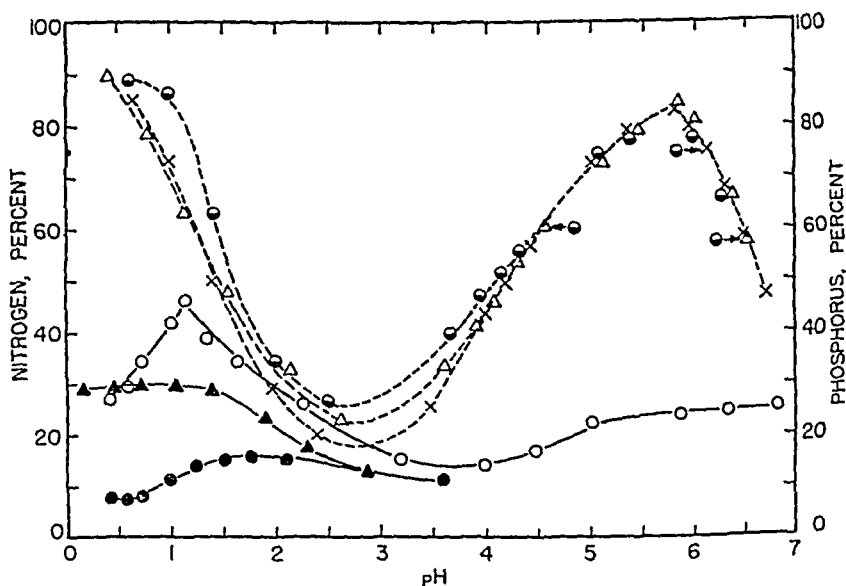


FIG. 5. The percentage of the total nitrogen and phosphorus of solvent-extracted cottonseed meal, which is soluble in hydrochloric, sulfuric, and trichloroacetic acid solutions at different pH values. HCl,  $\circ$  nitrogen,  $\times$  phosphorus;  $\text{H}_2\text{SO}_4$ ,  $\blacktriangle$  nitrogen,  $\triangle$  phosphorus;  $\text{CCl}_3\text{COOH}$ ,  $\bullet$  nitrogen,  $\bullet$  phosphorus.

acid used to adjust the pH, a finding which is in contrast to the phosphorus solubility behavior observed in the case of peanut meal with the same acids (Fig. 4). The small variation in phosphorus solubility, when these three acids are used to extract cottonseed meal, can be attributed to the fact that these acids differ less in their ability to peptize cottonseed meal proteins than in their ability to peptize peanut meal proteins in the pH range, 3.0 to 1.5. The influence of the solubility of nitrogenous constituents on the solubility of phosphorus compounds in seed meals is again apparent and points again to the existence of protein-phytic acid complexes in acid solutions.

The inorganic phosphorus content of the sulfuric and trichloroacetic acid extracts was, for all practical purposes, identical with that obtained by the use of hydrochloric acid, as is shown in Figs. 1 and 2 for peanut and cottonseed meals, respectively.

*Influence of Phytic Acid on Solubility of Peanut and Cottonseed Meal Proteins in Acid Solutions*—That phytic acid forms a complex with the proteins of peanut and cottonseed meal on the acid side of the isoelectric range has been demonstrated in the foregoing sections of this paper. It has been shown (16), also, that the removal of dialyzable meal constituents increases the solubility of the meal proteins in acids at pH values below their isoelectric range. Since the dialyzable meal constituents were found to contain a large percentage of the meal phosphorus, it was deemed probable that the removal of phytic acid through dialysis might be responsible for the increased solubility of the nitrogenous constituents of the dialyzed meal. Accordingly, experiments were designed to verify this assumption.

2.5 gm. samples of peanut and cottonseed meals were dialyzed against running distilled water at 4° for 48 hours. Dialysis resulted in the removal of 23 per cent of the meal solids, including 5 per cent of the nitrogen, 71 per cent of the ash, and 91 per cent of the phosphorus, from peanut meal and in the removal of 33 per cent of the cottonseed meal solids, including 7 per cent of the nitrogen, 65 per cent of the ash, and 67 per cent of the phosphorus. The dialyzed meal suspensions obtained in this manner were used to determine the effect of the addition of phytic acid on the solubility of the meal proteins. The nitrogen solubility of the dialyzed samples was determined under the following conditions. (a) The pH of individually dialyzed samples was adjusted with hydrochloric acid; (b) the pH was adjusted with hydrochloric acid, and 135 mg. and 260 mg. of sodium phytate<sup>4</sup> were added to the dialyzed peanut and dialyzed cottonseed meal suspensions, respectively; (c) the pH was adjusted with hydrochloric acid, and 110 mg. and 200 mg. of phytic acid<sup>4</sup> were added to the dialyzed peanut and

<sup>4</sup> The samples of sodium phytate and phytic acid were obtained through the courtesy of the Corn Products Refining Company, Argo, Illinois. Analysis by the authors

dialyzed cottonseed meal suspensions, respectively. The final volumes were brought to 100 ml. with distilled water. The results of analyses for soluble nitrogen are shown for peanut meal in Fig. 6 and for cottonseed meal in Fig. 7.

In Fig. 6 the difference between the nitrogen solubility curves for undialyzed and dialyzed peanut meals is apparent. The protein in the dialyzed meal is much more soluble between pH 4.0 and 1.5 than is that in the undialyzed meal, as is illustrated at pH 3.5 where the nitrogen solubility values are 75 and 15 per cent, respectively. Upon the addition of either sodium phytate or phytic acid in the concentrations previously specified, the nitrogen solubility values for the dialyzed meal in the pH range 4.0 to 1.5 are reduced to values even lower than were found for the undialyzed meal, indicating the formation of insoluble protein-phytic acid complexes. These lower nitrogen solubility values are due to the fact that, when sodium phytate or phytic acid was added to the dialyzed meal suspensions, the quantities added were equivalent to slightly more than the amount of phytic acid phosphorus lost on dialysis of the meal. To illustrate more clearly the formation of insoluble protein-phytic acid complexes, 500 mg. of sodium phytate were added to a dialyzed meal suspension at pH 1.4. Under these conditions 20 per cent of the total nitrogen was soluble, as compared to 80 per cent when only 135 mg. of sodium phytate were used.

Similar results were obtained when dialyzed cottonseed meal (Fig. 7) was subjected to the procedure described above for peanut meal. The addition of the specified amount of phytic acid did not produce as great a decrease in nitrogen solubility as did the sodium phytate, but this can probably be attributed to the impurity of the phytic acid. This is borne out by the fact that, when only 80 mg. of sodium phytate were added to the dialyzed meal suspension at pH 1.4, 50.5 per cent of the total nitrogen was soluble, whereas, when 260 mg. were added, only 29.3 per cent of the nitrogen was soluble at the same pH.

The results for both peanut and cottonseed meals show that the phytin present in these seed meals reduces the solubility of the meal proteins at pH values below the isoelectric points of the proteins. However, phytin does not appear to influence the solubility of peanut and cottonseed meal proteins at alkaline pH values, as is evidenced by the close similarity of the nitrogen peptization curves for the dialyzed and undialyzed meals in the alkaline range (16).

*Comparison of Nitrogen and Phosphorus Solubility Curves for Isolated*

revealed that less than 1 per cent of the total phosphorus was inorganic in the sodium phytate sample, and that the crude phytic acid sample contained 22 per cent total phosphorus, of which approximately one-fifth was inorganic phosphorus.

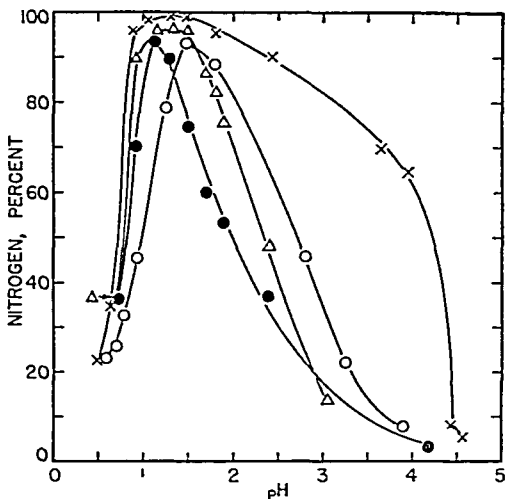


FIG. 6. The percentage of the total nitrogen of undialyzed (O) and dialyzed (X) peanut meal and of dialyzed peanut meal to which has been added either phytic acid ( $\Delta$ ) or sodium phytate ( $\bullet$ ), which is soluble in hydrochloric acid solutions at different pH values.

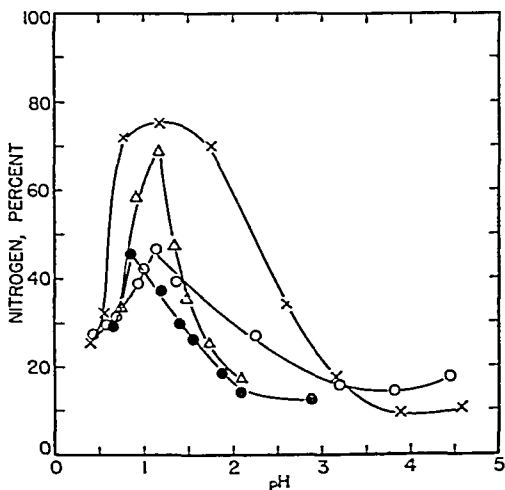


FIG. 7. The percentage of the total nitrogen of undialyzed (O) and dialyzed (X) cottonseed meal and of dialyzed cottonseed meal to which has been added either phytic acid ( $\Delta$ ) or sodium phytate ( $\bullet$ ), which is soluble in hydrochloric acid solutions at different pH values.

*Peanut and Cottonseed Protein and Their Significance in Protein Isolation*—Nitrogen and phosphorus solubility curves for peanut and cottonseed proteins containing 0.645 and 1.164 per cent total phosphorus, respectively, over a wide pH range (HCl and NaOH being used to adjust the pH) are shown in Figs. 8 and 9, respectively. The phosphorus solubility curves for the isolated proteins differ from the corresponding solubility curves for the meals in exhibiting (a) a broader minimum pH range of phosphorus solubility, (b) no decrease in the amount of soluble phosphorus at pH values above 6.0, and (c) a significantly lower phosphorus solubility in the pH range just

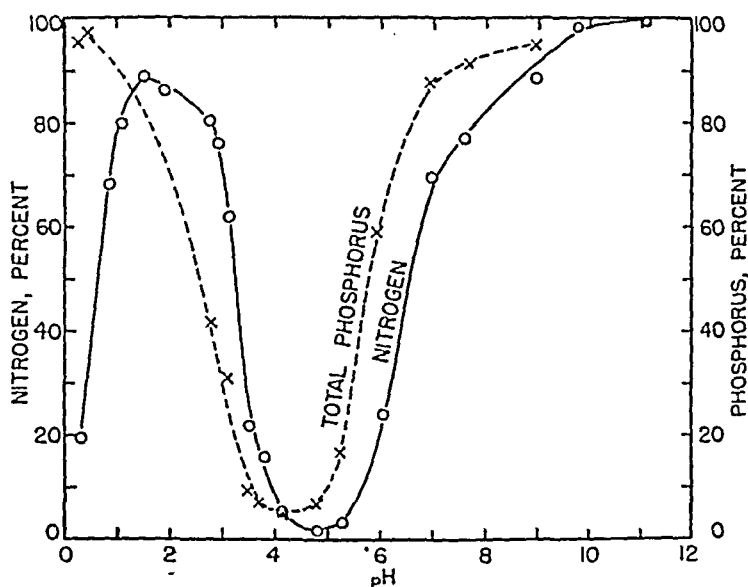


FIG. 8. The percentage of the total nitrogen and phosphorus of isolated peanut protein, which is soluble in hydrochloric acid and sodium hydroxide solutions at different pH values.

below the isoelectric zone of the proteins. There are, however, similarities in the phosphorus solubility curves for the isolated proteins and the corresponding meals; namely, (a) the phytic acid ion is displaced from the protein at very low pH values, and (b) on the alkaline side of the isoelectric zone of the proteins, the protein-phytic acid complexes are increasingly dissociated.

In order to interpret the differences and similarities that exist between the solubility curves for the isolated proteins (Figs. 8 and 9) and for the corresponding meals (Figs. 1 to 7), it is necessary to reconsider at this point some of the solubility data obtained on peanut and cottonseed meals. The progressive decrease in phosphorus solubility between pH 6.5 and 3.5

for peanut meal (Fig. 1), and between pH 6.0 and 2.75 for cottonseed meal (Fig. 2), may be attributed to the increased reactivity of the protein due to an increase in the positive charge on the protein molecules. Thus at pH 3.5 the proteins, representing approximately 50 per cent by weight of peanut meal, react with 85 per cent of the total meal phosphorus, amounting to a ratio of approximately 0.85 gm. of protein to 13.0 mg. of phosphorus. In other words, 1 gm. of the protein in peanut meal is capable of reacting at pH 3.5 with at least 15.3 mg. of phosphorus in the form of phytic acid. If a protein preparation could be isolated under

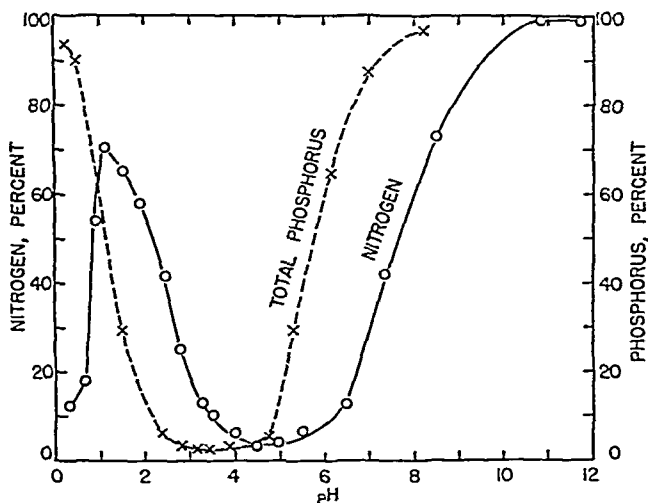


FIG. 9. The percentage of the total nitrogen and phosphorus of isolated cottonseed protein, which is soluble in hydrochloric acid and sodium hydroxide solutions at different pH values.

these conditions, it would contain phytic acid as an impurity to the extent of approximately 5.5 per cent of the weight of the protein. The closest experimental approach to the isolation of such a phytic acid-protein complex can be made by extracting the meal nitrogen at either pH 1.5 or 6.75, the two points where approximately maximum nitrogen and phosphorus solubility occur simultaneously, and by then precipitating the proteins at approximately pH 4.0 (Fig. 1).

Since cottonseed meal contains approximately twice as much phosphorus as peanut meal, it is possible for the cottonseed meal proteins to react with twice as much phytic acid as do the peanut meal proteins. Thus at pH 2.75 (Fig. 2) the proteins of cottonseed meal react with 82 per cent of the

total phosphorus, amounting to a ratio of approximately 0.82 gm. of protein to 25.2 mg. of phosphorus. 1 gm. of protein in cottonseed meal would, therefore, be capable of reacting at pH 2.75 with at least 30.8 mg. of phytic acid phosphorus. Such a protein preparation would contain approximately 11.0 per cent phytic acid as an impurity. The nitrogen and phosphorus solubility curves (Fig. 2) show, however, that the isolation of a major cottonseed protein fraction having such a high phytic acid content would be impossible if either hydrochloric acid or sodium hydroxide were used to extract the proteins from the meal. Nevertheless, it should be possible to obtain a protein fraction having a high phytic acid content by extracting cottonseed meal with a sodium chloride solution (0.5 N) at pH 6.0 (24) and by precipitating the protein at pH 2.75.

With these facts in mind it is possible to arrive at a clear interpretation of the solubility curves presented in Figs. 8 and 9 and to evaluate the significance of these results in protein preparation operations. The peanut and cottonseed protein preparations used in the experiments reported above can be used to illustrate some interesting points concerning the protein-phytic acid relationship in the peanut and cottonseed. It will be recalled that, in the preparation of the peanut protein, peanut meal was extracted at pH 8.0 and that under these conditions 96 per cent of the total meal nitrogen and 52 per cent of the total phosphorus were extracted (Fig. 1). Upon precipitation of the proteins at pH 4.5, approximately 90 per cent (Fig. 1) of the total meal nitrogen was precipitated. The percentage phosphorus in the isolated protein (after 3 days dialysis) was 0.645 per cent. The protein isolated under these conditions contained, therefore, 6.45 mg. of phosphorus per gm. of protein. Thus it is apparent that the protein reacted almost completely with the phytin which was present in the pH 8.0 meal extract, since only about 45 per cent of the total phytic acid phosphorus (equivalent to 3.43 mg. per gm. of meal) was extracted under these conditions. From a consideration of these data and the fact that phytic acid has been shown to diminish nitrogen solubility, it would be expected that the nitrogen solubility curve for isolated peanut protein should lie between the curves for the dialyzed meal (approximately 2 mg. of phosphorus per gm. of protein) and the undialyzed meal (approximately 15 mg. of phosphorus per gm. of protein), inasmuch as there is present in the isolated peanut protein sufficient phytic acid (approximately 6 mg. of phosphorus per gm. of protein) to reduce its solubility. This line of reasoning is partially substantiated by the nitrogen solubility behavior of isolated cottonseed protein. In this case, isolated cottonseed protein (approximately 11 mg. of phosphorus per gm. of protein) contains nearly the same ratio of phytic acid phosphorus to protein as does the dialyzed meal (approximately 10 mg. of phosphorus per gm. of protein) and, as would be



expected, the nitrogen solubility curves for the isolated protein and dialyzed meal are practically superimposable.

## DISCUSSION

### *Theoretical and Practical Significance of Protein-Phytic Acid Relationship*

The results reported in this paper on the solubility of the phosphorus and nitrogen compounds of peanut, cottonseed, and soy bean meals and the results of other investigators on oat flour (25), horse-bean meal (26), hemp meal (27), and wheat flour (28) suggest some interesting possibilities in protein chemistry which are of equal theoretical and practical importance. A consideration of these data reveals a possible reason for the difficulty encountered in the purification of some of the seed proteins and in obtaining protein preparations having reproducible physical and chemical characteristics.

In connection with a recent publication dealing with the color of peanut and cottonseed proteins (29), it was necessary to isolate a large number of protein preparations by different procedures. These and several additional preparations have since been analyzed for moisture, nitrogen, ash, and phosphorus, and representative results are given in Table I. The following conclusions may be drawn from these data: (a) For a given meal extract, the amount of phosphorus that will precipitate with the protein is dependent upon the pH of precipitation; (b) the phosphorus content of most of the protein preparations is not decreased appreciably by washing the moist protein cake with water or organic solvents; (c) the phosphorus content of an isolated protein preparation is practically independent of its ash content; (d) dialysis of aqueous suspensions of these protein preparations does not result in the removal of the phosphorus, although it has been shown earlier in this paper that, if dialysis of the meals is carried out at the proper pH values, appreciable amounts of the phosphorus can be removed.

It is apparent, therefore, that phytic acid is likely to be a major impurity in seed protein preparations. Jones and Csonka (30) isolated two protein fractions having high ash contents from a clear, filtered sodium chloride extract of cottonseed meal. The first protein fraction flocculated from the sodium chloride extract at 62°, and after its removal the second fraction was obtained by heating the solution at 85°. Since the nitrogen content of these two fractions was extremely low (approximately 2 per cent calculated on a moisture-free but ash-included basis), while the ash content (approximately 68 per cent on a dry basis) approached closely to that of phytin, it appears that the heating of the sodium chloride extract resulted in the flocculation of phytin with the occlusion of a small amount of protein.

Similar "impure phytin preparations" have been obtained from both peanut and cottonseed meals in the course of the present investigations.

TABLE I  
*Nitrogen, Phosphorus, and Ash Contents of Various Peanut and Cottonseed Protein Preparations*

Source and method of preparation*	Protein No.	Method of drying	Moisture	Ash	Nitrogen	Phosphorus
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Red skin peanuts; protein extracted, pH 7.0, pptd. at pH 6.0; washed 3 times with water	1	Alcohol-ether	10.2	0.2	15.3	0.21
	2	Air	10.5	0.2	14.9	0.24
Protein in supernatant from Nos. 1 and 2 pptd., pH 4.5; washed 3 times with water	3	Alcohol-ether	9.6	0.2	14.8	0.64
	4	Air	9.8	0.1	14.4	0.72
Portion pH 7.0 extract used for Nos. 1 and 2 adjusted to pH 4.5; washed 3 times with water	5	Alcohol-ether	9.4	0.2	15.0	0.52
	6	Air	9.3	0.3	14.9	0.70
Red skin peanuts; protein extracted, pH 8.2, pptd., pH 5.5; not water-washed	7	Air	9.5	1.45	15.0	0.30
	8	Dioxane	9.7	1.71	14.8	0.30
	9	Acetone	8.8	1.15	15.3	0.35
	10	MeEt ketone	9.7	1.27	15.3	0.33
Red skin peanuts; protein extracted, pH 8.2, pptd., pH 4.5; not water-washed	11	Air	9.3	1.33	14.2	0.85
	12	Dioxane	9.0	1.41	15.1	0.81
	13	Acetone	9.0	1.50	14.9	0.78
	14	MeEt ketone	8.9	1.48	15.0	0.79
White skin peanuts; protein extracted, pH 8.0, pptd., pH 4.5; dialyzed 72 hrs.	15†	Lyophilized	10.4	0.31	15.4	0.645
Petroleum ether-extracted cottonseed; protein extracted, pH 11.0, and pptd., pH 4.0; not water-washed	16	Air	9.3	3.35	13.0	1.14
	17	Dioxane	8.7	2.15	13.4	1.16
	18	Acetone	8.9	2.43	14.0	1.23
Ethyl ether-extracted cottonseed; protein extracted, pH 11.0, and pptd., pH 4.0; dialyzed 72 hrs.	19†	Lyophilized	10.6	0.22	14.0	1.164

\* A single batch of protein was prepared for each group, and the moist protein cake was divided into approximately equal parts. These were then dried as indicated.

† These protein preparations were those used in the present solubility investigation.

Vickery (31) has reported considerable difficulty in obtaining clear soy bean meal extracts that contain most of the meal nitrogen. The phytate ion may be partly responsible for this behavior, since Vickery's work was conducted at pH 7.1 to 7.5. Results obtained during experiments de-

signed to evaluate the color of various protein preparations (29) also suggest that the phytate ion may be responsible for the cloudiness of some protein solutions. It was found, in conducting color measurements, that practically all peanut protein preparations gave clear solutions upon moderate centrifugation at the high pH employed, whereas it was more difficult and often impossible to clarify solutions of soy bean and cottonseed protein preparations sufficiently to make spectrophotometric measurements possible. It was found that alkaline solutions of cottonseed protein preparations, after standing for some time, formed a flocculent precipitate which was high in phosphorus and probably consisted of phytin together with some adsorbed protein. Moreover, the ash content of the original protein preparation in such instances was relatively high. If, as these data seem to indicate, the phytate ion is responsible for the cloudiness of solutions of soy bean protein preparations, dialysis of a soy bean meal suspension against running distilled water at 4° should remove sufficient phytic acid and salts to make possible the subsequent preparation of a clear extract containing most of the protein nitrogen of the dialyzed meal:

Since phytic acid can be considered to occur as an impurity in most seed protein preparations, it is reasonable to suppose that its presence would influence the crystallization and denaturation behavior of these proteins and also influence the results of investigations that involve the measurement of the electrophoretic mobility, viscosity, or action of proteolytic enzymes on solutions of these proteins.

It should not be overlooked that phytic acid is a source of biologically active inositol which may be of commercial value either as a vitamin or as a starting material for the synthesis of organic chemicals. The seed meals, and particularly cottonseed meals, contain appreciable quantities of phytin which can be recovered under certain conditions as a by-product of protein isolation. Before the details of the protein-phytic acid solubility relationship were completed, it was thought that it might be possible to isolate phytin in good yields from the mother liquor remaining after the precipitation of the protein from seed meal extracts. However, the results given in this paper demonstrate that the isolation of phytin by this procedure is not economically or commercially feasible, inasmuch as the proteins, upon precipitation, combine with most of the phytic acid present in the extract. On the other hand, if peanut, cottonseed, and soy bean meals are leached with dilute acid at pH 5.5, 5.75, and 5.0 respectively, 80, 85, and 55 per cent, respectively, of the total phosphorus can be removed from these meals without an appreciable removal of protein nitrogen. The leachings could then be used for isolation of phytin, and the leached meals for protein isolation. As a matter of conjecture, protein preparations obtained from leached meals having low phytic acid content

might have sufficiently superior qualities for industrial utilization to justify this additional step in the isolation procedure.

#### SUMMARY

Solubility data are presented for the nitrogen- and phosphorus-containing constituents of peanut, cottonseed, and soy bean meals in various acid and sodium hydroxide solutions over a wide pH range.

It has been established that the naturally occurring phytic acid in seed meals is responsible for the suppression of the solubility of the seed meal proteins at pH values below their isoelectric points.

Phytic acid is a major impurity in isolated seed meal protein preparations, the amount depending upon the methods of extraction and precipitation and the degree of purification.

Evidence is presented which suggests that the phytate ion may influence the degree of clarity of certain seed meal extracts.

The approximate pH optima are given for the action of phytases in peanut, cottonseed, and soy bean meals.

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# DISTRIBUTION OF INTRAVENOUSLY INJECTED FRUCTOSE AND GLUCOSE BETWEEN BLOOD AND BRAIN\*

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Fructose, injected intravenously, in contrast to glucose, does not maintain the electrical activity of brain (2) or relieve symptoms of hypoglycemia (3) in eviscerated animals. However, the rate of respiration of brain slices in the presence of fructose does not differ significantly from the rate in the presence of glucose (4, 5) and the oxidation of fructose by broken cell preparations of brain follows the same pattern as for glucose (6). A hypothesis which would explain these findings is that the rate of transfer of fructose from blood to brain *in vivo* is not sufficient to provide a concentration of sugar that would meet the metabolic requirements of brain.

In the present work the distribution of fructose and glucose between arterial blood plasma and cerebral hemispheres of cats was determined at various times after intravenous injection of these sugars. The data obtained support the hypothesis.

## EXPERIMENTAL

In some experiments the cerebral hemispheres of the cats were widely exposed and the dura reflected; in others only the skull was exposed. The experiments were carried out under dial<sup>1</sup> (0.5 ml. per kilo, one-half intraperitoneally, the rest intramuscularly) or sodium amytal (0.07 gm. per kilo subcutaneously) anesthesia. The distribution of sugar between blood and brain was found not to be an obvious function of the different operations or anesthetics.

About 1 hour after the operation an initial sample of blood from the femoral artery was collected in a mixture of fluoride and oxalate or in heparin. The sugars, in a concentration of 0.5 gm. per ml., were injected into the femoral vein at a rate of about 3 ml. per minute. After injection of sugar, additional blood samples were collected from time to time. A final sample of blood was collected at the time the brain was frozen by application of liquid air. The hemispheres were chiseled out and ground

\* A preliminary report has been presented (1).

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<sup>1</sup> 0.1 gm. of diallylbarbituric acid and 0.4 gm. each of urethane and monoethylurea per ml. The laboratory is indebted to Ciba Pharmaceutical Products, Inc., for a supply of this drug.

in a mortar while frozen. Aliquots of tissue were assayed for sugar and blood.<sup>2</sup> All samples of blood, and plasma from the initial and final samples of blood, were assayed for sugar. Plasma from heparinized blood was prepared as quickly as possible. The sugar content of such plasma did not differ significantly from plasma prepared from blood collected in fluoride and oxalate.

Protein-free filtrates of blood, plasma, and tissue were prepared with zinc hydroxide (7). Fructose was estimated colorimetrically (8). Glucose and total sugar, *i.e.* glucose plus fructose, were analyzed colorimetrically with a copper reagent (7). Since the degrees of reduction of the copper reagent by fructose and glucose were found to be practically identical, the difference between total sugar and fructose was considered a measure of glucose. The concentration of blood in brain was estimated by comparison of the concentration of hemoglobin in blood and brain (9).

Blood, plasma, and brain from animals not given fructose were found to yield some color in the assay for fructose.<sup>3</sup> In the case of brain this color was equivalent to  $5 \pm 1$  mg. per 100 gm. The average value, 5 mg. per 100 gm., was applied as a correction in the assay of brain from animals given fructose. In the cases of blood and plasma the values obtained with the preinjection samples were used as corrections. These corrections did not exceed 5 mg. per 100 ml.

### Results

The data are presented in Table I.

When no sugar was injected (Experiments 1 to 6), the mean ratio of brain to plasma glucose and its standard deviation were  $0.411 \pm 0.029$ . Following injection of glucose (Experiments 7 to 13), the ratios of tissue to initial plasma glucose increased, the lowest ratio being 0.70, the highest 1.28. Comparison of these several ratios indicates that injection of glucose was followed by an increase in brain glucose. The increments in brain glucose were calculated as follows: The product of the mean ratio given above and the preinjection levels of plasma glucose in a given case represents the initial level of tissue glucose. The difference between the observed level of tissue glucose and the calculated initial level is a measure of the increment that followed injection. The results of the calculations are given in Table I. It may be noted that the increases in plasma glucose following injection of fructose were accompanied by increases in tissue glucose.

Following injection of fructose (Experiments 14 to 22), this sugar was

<sup>2</sup> About 0.06 ml. of blood per gm. of brain was found in the experiments in which the hemispheres were exposed and about 0.02 ml. per gm. in those in which only the skull was exposed.

<sup>3</sup> In the preliminary report (1) this finding was not taken into account.



found in brain. The highest concentration of fructose (Experiment 22) was about two-thirds of the lowest concentration of glucose (Experiment 4) and about two-thirds of the smallest increment in tissue glucose (Experiment 7).

TABLE I

*Distribution of Intravenously Injected Fructose and Glucose between Plasma and Cerebral Hemispheres of Cats*

The concentrations of the sugars in tissue are given in terms of blood-free material. The increase in tissue glucose was calculated as indicated in the text.

Experiment No.	Sugar	Dose	Time between 1st injection of sugar and collection of final blood and tissue	Glucose				Fructose	
				Plasma		Tissue		Plasma	Tissue
				Preinjection	Final	Observed	Increase		
		gm. per kg.	min.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 ml.	mg. per 100 gm.
1					183	72			
2					253	120			
3					127	51			
4					100	39			
5					170	72			
6					110	43			
7	Glucose	1	5	100	527	82	41		
8	"	1	7	147	577	128	68		
9	"	1	26	127	472	136	84		
10	"	2	7	87	930	104	68		
11	"	2	18	256	820	178	73		
12	"	2	24	127	660	162	110		
13	"	3*	69	212	824	223	136		
14	Fructose	1	8	178	213	93	20	200	3
15	"	1	10	306	380	140	14	212	6
16	"	1	30	167	220	81	12	236	3
17	"	1.5	20	119	213	64	15	310	0
18	"	2	5	298	270	131	9	980	0
19	"	2	11	358	345	180	33	765	0
20	"	2	26	288	214	123	5	596	13
21	"	2.9	32	245	306	132	31	324	13
22	"	3*	64	247	362	132	30	275	25

\* 2 gm. per kilo were given initially. About 30 minutes later an additional 1 gm. per kilo was given.

The highest level of fructose was reached in about 1 hour after injection of the sugar, while the smallest increment in glucose was attained in 5 minutes.

The rate of oxidation of fructose by brain *in vitro* is certainly not greater than the rate of oxidation of glucose (4, 5) and under certain conditions is less (10). It may be presumed, therefore, that the observed differences in the increments of glucose and fructose are attributable to differences in their

rates of transfer from blood to brain. Comparison of Experiment 13 with 22 and of Experiment 7 with 14 indicates that the rate of transfer of glucose was 5 to 10 times greater than that of fructose.

#### DISCUSSION

The data of Kerr, Hampel, and Ghantus (11) indicate that severe symptoms of hypoglycemia appeared in cats when the concentration of glucose in brain was 26 mg. per 100 gm. or less. In the present experiments it was found that the highest level of brain fructose, 25 mg. per 100 gm., was obtained about 1 hour after injection of the sugar and that appreciable levels were obtained in some experiments about  $\frac{1}{2}$  hour after injection. Such levels were attained only after injection of 2 or more gm. of sugar per kilo. It seems reasonably certain, therefore, that the rate of transfer of fructose from blood to brain is not sufficient to provide a concentration that might be expected to prevent or relieve the effects of hypoglycemia in eviscerated animals.

#### SUMMARY

The concentrations of fructose and glucose in blood plasma and brain, determined at intervals after intravenous injections of these sugars, are such as to indicate that the rate of transfer of fructose from blood to brain is considerably less than that of glucose. The concentrations of fructose found in brain were less than the concentrations of glucose required to maintain normal central nervous function.

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# PHOSPHORIC ESTERS OF BIOLOGICAL IMPORTANCE

## I. THE SYNTHESIS OF GLUCOSE-6-PHOSPHATE

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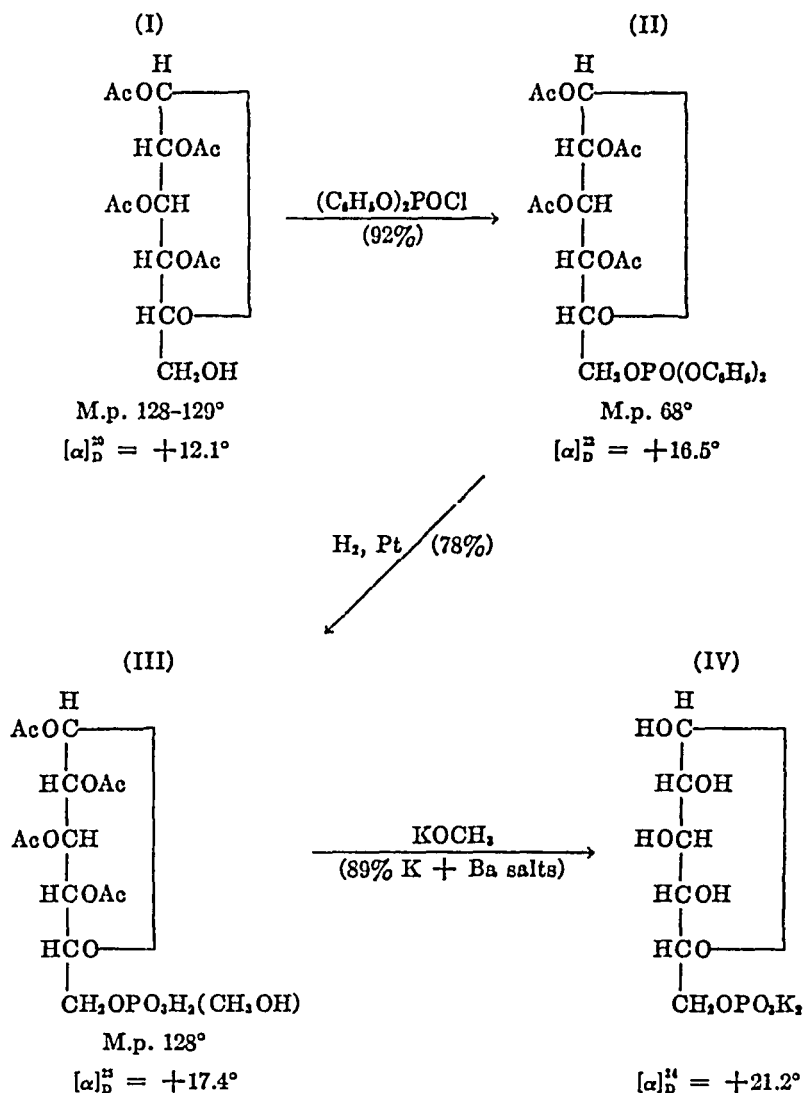
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Previous methods of preparing glucose-6-phosphate for use in biological experimentation were based on the isolation of this ester from a crude mixture of hexose monophosphates, obtained by yeast fermentation. The isolation was achieved by fractional crystallization of the brucine salts (1) or by preferential hydrolysis of the fructose component which leaves a considerable portion of the aldose phosphate intact (2). It has also been prepared by allowing phosphoglucomutase to act on glucose-1-phosphate (3). A chemical synthesis of the Robison ester was reported by Levene and Raymond (4), who treated monoacetone glucose with phosphorus oxychloride in pyridine at low temperatures. The yield of barium salt was only fair and it was necessary to purify the product by several recrystallizations of the brucine salt before it exhibited the proper rotation. These workers also attempted to phosphorylate glucose-1,2,3,4-tetraacetate but obtained an impure product in very poor yield.

The necessity of obtaining quantities of pure glucose-6-phosphate for enzymatic investigations prompted an attempt to devise a practical synthesis of the compound. The steps involved are shown in the accompanying diagram. Advantage was taken of the use of diphenylchlorophosphonate (5) as a phosphorylating agent. This substance reacts with only 1 mole of the substance to be phosphorylated, does not cause the formation of chlorohydrin compounds, as does  $\text{POCl}_3$  (6), and the substituted diphenylphosphoric esters formed usually lend themselves to purification by recrystallization more readily than the products obtained when phosphorus oxychloride is employed. The phenyl groups are removed readily by reductive cleavage with hydrogen in the presence of platinum oxide catalyst.

The position of attachment of the phosphate group to the glucose molecule was insured by using as a starting material 1,2,3,4-tetraacetyl- $\beta$ -D-glucopyranose (7, 8), in which carbon atom 6 has the only free hydroxyl group. The original procedure for the preparation of 1,2,3,4-tetraacetyl- $\beta$ -D-glucopyranose (7) has been improved by Reynolds and Evans (8), but

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in an earlier study (9), in which this glucose tetraacetate was used in the synthesis of gentiobiose for biological experimentation, some difficulty was experienced in obtaining consistently good yields by their crystallization procedures. It has now been found that dibutyl ether is more reliable than either ethyl ether or petroleum ether for the crystallization of the tetraacetate and yields a product of sufficient purity for phosphorylation without recrystallizing.

After the reductive cleavage of the phenyl groups from the phosphory-

lated glucose tetraacetate, the acetyl groups were removed by catalytic saponification with potassium methylate in anhydrous methanol. The potassium salt of glucose-6-phosphate was precipitated from the solution as the deacetylation proceeded; it was purified by washing several times with anhydrous methanol and dried *in vacuo* over  $P_2O_5$ . The acetyl groups could also be removed by acid in aqueous solution, or by the slow addition of the stoichiometric amount of sodium or potassium hydroxide solution to an alcoholic solution of the ester, and the product was then isolated as the barium salt. When the acetyl groups were removed with aqueous alkali, some discoloration occurred (removable with charcoal) and the final product contained appreciable quantities of fructose-6-phosphate, the presence of which was indicated by optical rotation, the quantitative Seliwanoff test, and enzymatically catalyzed equilibria. For biological purposes the potassium salt has the advantage that it is readily soluble in water and may be used directly without the inconvenience of removing the cation.

The purity of the glucose-6-phosphate was determined not only by analyses and optical rotation but also by *quantitative* enzymatic studies. Synthetic glucose-6-phosphate was converted by phosphohexoisomerase to the same equilibrium (32 per cent fructose-6-phosphate to 68 per cent glucose-6-phosphate) as was pure fructose-6-phosphate, prepared according to Neuberg, Lustig, and Rothenberg (10).

When calculated on the basis of the anion portion of the salts, the optical rotation of our potassium salt is almost exactly that of the calculated value ( $[\alpha]_{541} = [\alpha]_D \times 1.18$  (1)) for Robison's purest barium salt obtained from the several times recrystallized brucine salt of natural aldose monophosphate.

The barium salt of 6-phosphogluconic acid has been prepared from synthetic glucose-6-phosphate by the procedure of Robison and King (1).

#### EXPERIMENTAL

*1,2,3,4-Tetraacetyl- $\beta$ -D-glucopyranose (I)*—This starting material was prepared from 6-trityltetraacetyl- $\beta$ -D-glucose, according to the procedure of Helferich and Klein (7), except that the crystallization was made from a concentrated chloroform solution (not sirup) by the slow addition of dibutyl ether. The first crop of crystalline tetraacetyl- $\beta$ -D-glucopyranose corresponded to a yield of 67 per cent and had a melting point of 124–127°. This material was phosphorylated either directly or after recrystallizing from chloroform by the addition of dibutyl ether which gave the pure substance, m.p. 128–129°.

*1,2,3,4-Tetraacetyl-6-diphenylphosphono- $\beta$ -D-glucopyranose (II)*—To a cooled solution of 7.1 gm. of 1,2,3,4-tetraacetyl- $\beta$ -D-glucopyranose in 20 cc. of anhydrous pyridine, 6.0 gm. of diphenylchlorophosphonate (5) were

added dropwise with continuous shaking and cooling in an ice bath. The reaction began at once and within a few minutes a copious crystalline precipitate of pyridine hydrochloride appeared. The mixture was kept in the ice bath for 15 minutes and then placed in a refrigerator at 10° overnight. A few drops of ice water were added to hydrolyze the excess of acid chloride and after one-half hour the product was separated by pouring slowly into 600 cc. of ice water under continuous stirring. When the precipitate became granular, it was filtered off and again stirred up in fresh ice water. The product was filtered off, washed with cold water, and dissolved in 100 cc. of chloroform. The chloroform solution was washed once with dilute HCl and two times with distilled water, was dried with anhydrous sodium sulfate, and evaporated under reduced pressure to a sirup. The product was crystallized by careful addition of petroleum ether (b.p. 60–80°), swirling, and allowing to stand for several hours; the process may be hastened by seeding or scratching. The product was filtered with suction, washed with petroleum ether, and dried. Yield, 10.9 gm. (92 per cent of the theoretical). It melted at 64–66° and was of sufficient purity for subsequent use. The pure substance may be obtained by recrystallizing from isopropyl ether, or from acetone by the addition of water, m.p. 68°. It is soluble in chloroform, acetone, benzene, and ethyl alcohol.  $[\alpha]_D^{22} = +16.5^\circ$  ( $c = 1.37$  in anhydrous pyridine).

$C_{26}H_{41}O_{13}P$ (580.5).	Calculated.	C 53.8,	H 5.04,	P 5.34
	Found.	" 53.8,	" 5.03,	" 5.24
		" 53.8,	" 5.03,	" 5.32

*1,2,3,4-Tetraacetyl-β-D-glucose-6-phosphoric Acid (III)*—A solution of 7.0 gm. of tetraacetyl-6-diphenylphosphono-β-D-glucopyranose (II) in 70 cc. of anhydrous methanol<sup>1</sup> was shaken in an atmosphere of pure dry hydrogen at a pressure slightly greater than 1 atmosphere with 0.7 gm. of platinum oxide (Adams' catalyst). When the reduction neared completion, the free acid began to crystallize in fine needles. The absorption of hydrogen stopped abruptly when the theoretical quantity (8 moles) had been consumed; this required from 2.5 to 4.5 hours in several runs. After warming to dissolve the product, the catalyst was removed by filtering or centrifuging. An equal volume of petroleum ether was added in portions to the filtrate and crystallization allowed to proceed during slow cooling. The crystals were filtered with suction, washed with petroleum ether, and dried *in vacuo* at room temperature. Yield, 3.6 gm. (65 per cent of the theoretical). The product melted at 126–128°, and contained the theoretical quantity of organic phosphorus. When recrystallized from anhydrous methanol by slow addition of petroleum ether, the substance melted

<sup>1</sup> Prepared according to Lund and Bjerrum (11).

at 127–128°. A second crop of crystals of the original purity may be obtained by evaporating the mother liquors to dryness under reduced pressure at a bath temperature of 25° and recrystallizing the product from methanol-petroleum ether. The analyses indicated that the substance crystallized with 1 mole of methanol which could not be removed by heating *in vacuo* without causing further decomposition. It was demonstrated by electrometric titration that the methanol was not esterified with the phosphoric acid residue. The presence and identity of the methanol were established by converting it to methyl iodide, which was trapped in dimethylalanine (12). The trimethylphenylammonium iodide obtained (a) melted at 227°, that from  $\alpha$ -methyl glucoside (b) melted at 227°, that from pure methanol (c) melted at 230°, and mixtures of (a) and (c) melted at 227°. Phillips (13) reported the melting point of trimethylphenylammonium iodide to be 231.6°.

$[\alpha]_D^{25} = +17.4^\circ$  ( $c = 1$  (of methyl alcoholate) in anhydrous pyridine); calculated for solvent-free compound  $[\alpha]_D^{25} = +18.7^\circ$ .

$C_{11}H_{21}O_{11}P$ (428.3).	Calculated. C 39.3, H 4.94, P 7.23
$C_{11}H_{21}O_{11}P + CH_3OH$ (460.3).	“ “ 39.1, “ 5.47, “ 6.73
	Found. “ 39.2, “ 5.63, “ 6.7

*Reduction in Anhydrous Ethanol*—The reductive cleavage of 3.8 gm. of the diphenyl compound (II) was carried out in 25 cc. of anhydrous ethanol with 0.4 gm. of platinum oxide. The product, which crystallized from the solvent as the reduction proceeded, was so sparingly soluble in hot ethanol that additions of anhydrous acetone were required to separate it from the catalyst. Slow evaporation of the solvents under reduced pressure caused the product to crystallize. After filtering and drying *in vacuo* over  $CaCl_2$  and paraffin, the product weighed 2.42 gm. (78 per cent of the theoretical) and melted at 126–127°. It was recrystallized from anhydrous ethanol and dried as above for analysis.

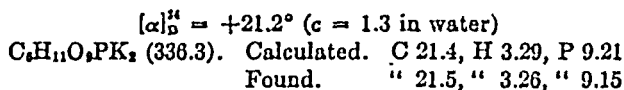
The solvent of crystallization was characterized by distillation (at the melting point of the compound) into a pyridine solution of 3,5-dinitrobenzoyl chloride, isolation, and recrystallization of the ester formed; m.p. 93°, authentic ethyl 3,5-dinitrobenzoate melted at 93°, mixed m.p. 93°.

$[\alpha]_D^{25} = +16.9^\circ$  ( $c = 1$  (of ethyl alcoholate) in anhydrous pyridine); calculated for solvent-free compound  $[\alpha]_D^{25} = +18.7^\circ$ .

$C_{11}H_{21}O_{11}P + C_2H_5OH$ (474.4).	Calculated. C 40.5, H 5.74, P 6.53
	Found. “ 40.6, “ 5.57, “ 6.44
	“ 40.6, “ 5.67

*Potassium Glucose-6-phosphate (IV)*—To 3.3 gm. of tetraacetylglucose-6-phosphoric acid (III) (from methanol) partially dissolved in 75 cc. of cold

anhydrous methanol, a sufficient quantity of potassium methoxide in anhydrous methanol<sup>2</sup> to neutralize the free acid groups was added dropwise with shaking. Complete solution was attained after the first few drops were added. Cleavage of the acetyl groups was initiated by the addition of a catalytic excess of 1.5 milliequivalents of potassium methoxide. The potassium salt of glucose-6-phosphate began to separate at once. The cleavage was allowed to proceed at refrigeration temperature in a tightly stoppered flask overnight. The product was separated by centrifuging, was washed four times with anhydrous methanol, once with each of the following methanol-ether mixtures: 80:20, 50:50, 20:80, and twice with anhydrous ethyl ether. After drying *in vacuo* at room temperature, the product weighed 1.65 gm. (68.5 per cent of theory). It was essential to use only anhydrous solvents and thoroughly dried equipment in order to obtain good yields.



From the combined mother liquor and methanol washings an additional quantity of glucose-6-phosphate was obtained as the barium salt. The slightly turbid alcohol solutions were treated with an excess of  $BaBr_2$  in anhydrous methanol. When the barium salt had settled, it was separated by centrifuging, washed with absolute alcohol, and finally with ether. After purification, as described in the following section, the barium salt weighed 0.6 gm. (21 per cent of original theory); thus the combined yield of potassium and barium salts was 89.5 per cent of theoretical. To obtain all of the product as the barium salt,  $BaBr_2$  solution was added after the deacetylation by potassium methoxide was completed, and the barium salts were again purified, as described in the following section.

*Deacetylation with Acid*—0.5 gm. of tetraacetylglucose-6-phosphoric acid (III) was dissolved in 35 cc. of 0.66 N HBr and the solution was heated on the steam bath for 3 hours. After cooling, pulverized barium hydroxide was added to neutrality. The solution was filtered and 4 volumes of ethanol were added. When the precipitate had settled, the supernatant liquor was decanted. The precipitate was washed in succession with 90 per cent ethanol, absolute ethanol, 75 per cent ethanol-25 per cent ether, 25 per cent ethanol-75 per cent ether, and finally with dry ether. After drying in air, the barium glucose-6-phosphate was dissolved by extracting successively with 20, 10, and 5 cc. portions of distilled water. To the clear filtrate 4 volumes of ethanol were added and the product was separated

<sup>2</sup> Prepared by the cautious addition of clean potassium metal to anhydrous methanol. Solutions of 1 to 2 N were used.



and dried as above. The barium salt (0.33 gm.) was free of inorganic phosphate and on the basis of its organic phosphorus content was 93 per cent pure (yield = 72 per cent of theory). Its rotation (purity based on phosphorus analysis) was  $[\alpha]_D^{24} = +17.9^\circ$ .

**Biological Activity**—The following data indicate that the synthetic glucose-6-phosphate is quantitatively biologically active. A partially purified preparation of phosphohexoisomerase (free of phosphoglucomutase activity) from rat muscle converted 32 per cent of the synthetic ester to fructose-6-phosphate at equilibrium at  $25^\circ$ . Under the same conditions, pure fructose-6-phosphate, prepared according to Neuberg, Lustig, and Rothenberg (10), was converted to an equilibrium of 68.4 per cent glucose-6-phosphate to 31.6 per cent fructose-6-phosphate. Fructose phosphate was determined in these experiments by a quantitative Seliwanoff test (2).

#### SUMMARY

A new procedure is described for the synthesis of glucose-6-phosphate. 1,2,3,4-Tetracetyl- $\beta$ -D-glucopyranose has been phosphorylated with diphenylchlorophosphonate in pyridine. Subsequent removal of the phenyl groups by means of hydrogen and platinum oxide, followed by saponification of the acetyl groups, gave pure glucose-6-phosphate in good yield. The ester was isolated as a crystalline dipotassium salt which is readily soluble in water and can be used directly for enzymatic experimentation. The barium salt may be prepared by an alternative procedure.

The synthetic ester is a convenient source of 6-phosphogluconic acid.

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# REACTION OF THIOL COMPOUNDS WITH PEROXIDASE<sup>\*</sup> AND HYDROGEN PEROXIDE<sup>\*</sup>

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Recent studies have indicated that peroxidase might function in the biological synthesis of thyroxine by the thyroid gland. Johnson and Tewkesbury (1) believed that thyroxine might be formed by the oxidative coupling of 2 molecules of diiodotyrosine. Westerfeld and Lowe (2) studied the oxidative condensation of *p*-cresol by hydrogen peroxide and peroxidase and suggested that peroxidase might be involved in the biological synthesis of thyroxine by a similar mechanism. Keston (3) intimated that hydrogen peroxide may be produced in living cells by the flavoprotein systems and that the hydrogen peroxide and peroxidase accelerated the iodination of protein *in vitro*. The observation that thiourea inhibited the staining of thyroid tissue by the peroxidase reagent, benzidine-hydrogen peroxide, led Dempsey (4) to propose that the antiperoxidase activity of thiourea might account for its goitrogenic activity. Glock (5) further observed that thiourea and thiouracil inhibited the action of peroxidase and hydrogen peroxide on pyrogallol; however, she found only insignificant amounts of true peroxidase in thyroid gland and considered, therefore, that the goitrogenic activity of thiourea was not due to its antiperoxidase activity. Franklin *et al.* (6, 7) demonstrated that oxidative mechanisms were necessary for the incorporation of iodide into thyroxine by the thyroid gland. This iodination was prevented by thiouracil as well as by oxidative inhibitors. Thiourea has the properties of an antioxidant in the prevention of the discoloration of fruit (8) and in the prevention of peroxide formation in fats (9).

The above observations suggested that the antioxidant properties of the thiol compounds might be related to their antithyroid activities. It was considered further that the antiperoxidase activities of the thiol compounds might be a measure of their antioxidant properties. Accordingly, a number of thiol compounds were tested for their antiperoxidase activities.

*Antiperoxidase Activities*—Lipmann's method (10) was used for measuring the inhibitory effect of thiols on horseradish peroxidase. In this method the amount of red dye produced by the action of peroxidase and hydrogen

<sup>\*</sup> An abstract of this investigation has been published (*Federation Proc.*, 5, 501 (1946)).

peroxide on *p*-aminobenzoic acid was measured colorimetrically. It was observed that thiourea inhibited the peroxidase activity by 50 per cent at an average concentration of 0.00043 M; 2-thiouracil was twice as active and 2,6-dithiouracil was 4 times as active as thiourea. A series of thirty-five thiourea derivatives (11) had antiperoxidase activities varying from one-fifth to 6 times that of thiourea, *o*-phenetylthiourea having the greatest activity. Sulfanilamide and sulfathiazole were one-tenth as active as thiourea. Cysteine was equally active, while glutathione was 3 times as active as thiourea. These results apparently confirmed the antiperoxidase activities of thiourea and thiouracil, the sulfonamides, and cysteine and glutathione as observed by Glock (5), Lipmann (10), and Balls and Hale (12), respectively. However, further work has now shown that the apparent antiperoxidase activities of thiol compounds are due to their reducing action on the peroxidase system.

Within the series of thirty-five thiourea derivatives there appeared to be a rough correlation of the antiperoxidase activity and the antithyroid potency as assayed by the increase in weight of rat thyroid glands (unpublished experiments). Dithiouracil was more potent than thiouracil and the latter more active than thiourea. The sulfonamides had low antiperoxidase activities and also low antithyroid potencies (13). However, a number of thiol derivatives had high antiperoxidase activities but no antithyroid activities; *e.g.*, cysteine, glutathione, and sodium sulfide.

*Reducing Power of Thiol Compounds*—Elliott (14) had observed that sulfhydryl compounds were capable of reducing the colored dyes formed by the action of peroxidase and hydrogen peroxide on various substrates, such as benzidine, guaiacol, and *p*-phenylenediamine. It was considered, therefore, that the apparent antiperoxidase activities of thiols might be due to their reducing action on the red dye (PABA red) formed by peroxidase and hydrogen peroxide from *p*-aminobenzoic acid. To test the reducing power of the thiols they were added in 0.001 M concentration at pH 7.0 to aliquots of PABA red and benzidine blue. The latter were made by reaction of hydrogen peroxide and peroxidase with *p*-aminobenzoic acid and benzidine and removal of the excess hydrogen peroxide with catalase. Also, the reducing power of thiols was tested against 2,6-dichlorophenol indophenol by measuring the decoloration of the dye in evacuated Thunberg tubes. The thiols were added at 0.001 M concentration to the dye (0.025 per cent) at pH 7.0. The reducing action of the various thiols on the dyes is recorded in Table I.

It was observed that thiourea and *o*-phenetylthiourea did not reduce the red dye, PABA red. However, the other thiols tested were able to reduce the PABA red dye. All of the thiols tested decolorized the benzidine blue. The fact that thiouracil has the power of decolorizing benzidine blue could

partially explain the inhibition of the benzidine blue staining reaction of thyroid tissue by thiouracil, as observed by Dempsey (4). Consequently, thiouracil might not have any action on peroxidase at all but might show an apparent inhibition by reducing the colored compound formed by peroxidase and hydrogen peroxide.

The observation that all of the thiol compounds reduced the dye, 2,6-dichlorophenol indophenol, at pH 7.0, indicated that they had reducing potentials more negative than +0.217 volt (15). Balls and Hale (16) observed that most substrates of peroxidase and hydrogen peroxide were capable of reducing this dye.

*Mechanism of Action of Thiols with Peroxidase and Hydrogen Peroxide*—Since some thiols were found to have the power of decolorizing PABA red and benzidine blue, indicating that their apparent antiperoxidase activities might be due to their reducing powers rather than to a true inhibitory action

TABLE I  
*Reducing Action of Thiols on Dyes*

Compound	<i>p</i> -Aminobenzoic acid red	Benzidine blue	2,6-Dichlorophenol indophenol
Thiourea.....	—	+	+
<i>o</i> -Phenethylthiourea.....	—	+	+
2-Thiouracil.....	+	+	+
2,6-Dithiouracil.....	+	+	+
Glutathione.....	+	+	+
Cysteine.....	+	+	+

on peroxidase, it was desired to test the action of thiols on the peroxidase system by a method which did not depend on the formation of a colored complex. The method of Balls and Hale (12) was tried in which the rate of disappearance of hydrogen peroxide in the presence of peroxidase and a suitable substrate is measured. By this method the amount of hydrogen peroxide remaining after it had acted on a substrate for a given time was determined by titrating the iodine liberated from potassium iodide with thiosulfate. It was found that this method could not be used for measuring the effect of thiols on peroxidase, because the thiols reacted with the iodine liberated from potassium iodide by hydrogen peroxide. This fact explains the apparent antiperoxidase activities of cysteine and glutathione observed by Balls and Hale (17). Campbell *et al.* (18) observed that thiourea was oxidized by iodine to formamidine disulfide hydriodide.

Elliott's (14) semiquantitative gasometric method for measuring the rate of disappearance of hydrogen peroxide was then tried. In this method, manganese dioxide was used to liberate oxygen from hydrogen peroxide and

the volume of gas was measured. It was found that thiol compounds react with the manganese dioxide; therefore, this method was not applicable.

A manometric method was developed for determining peroxidase activity by measuring the rate of disappearance of hydrogen peroxide in the presence of peroxidase and a suitable substrate. Catalase was used to liberate oxygen from the hydrogen peroxide remaining after a given time and the volume of oxygen was measured with the Warburg apparatus. The peroxidase, prepared from horseradish by Elliott's method (14), had 0.55 purpurogallin unit per ml. Catalase was prepared from beef liver by Sumner's method (19). The reaction vessels contained in the reaction chamber 1.0 ml. of peroxidase, 0.5 ml. of various concentrations of the substrate, *p*-aminobenzoic acid, and 0.5 ml. of 0.1 M phosphate buffer at pH 7.0; in one side arm was placed 1.0 ml. of 0.015 M hydrogen peroxide and in the second side arm, 0.5 ml. of catalase. After equilibration at 38°, the hydrogen peroxide was added to start the reaction. After 5 minutes, catalase was added from the second side arm to stop the reaction and liberate oxygen from the hydrogen peroxide. The volume of oxygen was measured and the amount of hydrogen peroxide calculated as usual. The amount of hydrogen peroxide initially present was determined with a separate vessel in which the substrate was omitted. The rate of peroxidase activity was, therefore, measured by the rate of disappearance of hydrogen peroxide. The effects of thiols on peroxidase activity were studied by measuring the rate of disappearance of hydrogen peroxide in the presence and absence of the thiols.

The principles of Lineweaver and Burk (20) were used to design an experiment to examine the nature of reaction between thiourea and the peroxidase system. The rate of reaction of the peroxidase system was determined with various concentrations of the substrate, *p*-aminobenzoic acid. The effect of thiourea at various concentrations was then determined at each substrate concentration.

The reciprocals of the substrate concentrations and the reciprocals of the rates of utilization of hydrogen peroxide were plotted in Fig. 1. The best straight lines were calculated by the method of least squares. The deviations from linearity were not statistically significant. The linear relationship between the reciprocals of the substrate concentration and the rates of utilization of PABA demonstrated the applicability of the Lineweaver and Burk equation to the reaction between PABA and peroxidase and hydrogen peroxide. The effects of thiourea were surprising in that the slopes were decreased with increasing concentrations of thiourea, while the intercepts were increased. The dissociation constants consequently decreased with increasing thiourea concentration.

This experiment was interpreted as indicating that there was no inhibition of peroxidase by thiourea at all, but, on the contrary, an acceleration of the rate of utilization of hydrogen peroxide. It was evident, therefore, that

there was a competition between thiourea and PABA for the available peroxidase and hydrogen peroxide. Thiourea was evidently a substrate for the peroxidase system just as PABA was a substrate.

The reaction between thiols and hydrogen peroxide in the presence and absence of peroxidase was then measured manometrically. The reaction vessels contained the following reactants: 1.0 ml. of 0.0075 *M* thiol, 0.5 ml. of 0.1 *M* phosphate buffer, pH 7.0, 1.0 ml. of water or peroxidase, 1.0 ml. of 0.015 *M* hydrogen peroxide, and 0.5 ml. of catalase. After equilibration at 38°, the reaction was started by adding hydrogen peroxide from the side

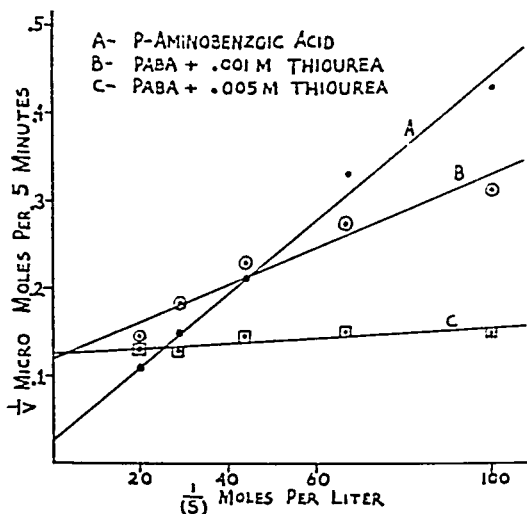


FIG. 1. Effect of thiourea on the rate of oxidation of *p*-aminobenzoic acid by hydrogen peroxide and peroxidase. The reciprocal substrate concentration is plotted against the reciprocal velocity.

arm and stopped at intervals by adding catalase from the second side arm. The moles of hydrogen peroxide used per mole of thiol present were calculated. The data are illustrated in Fig. 2.

The results demonstrated that thiols reacted with hydrogen peroxide at measurable rates and that peroxidase accelerated the reaction. Therefore, the thiols are substrates for the peroxidase-hydrogen peroxide system.

An indication of the extent of the reactions is obtained from the quantities of hydrogen peroxide which reacted with the thiols. 2 moles of thiourea could react with 1 mole of hydrogen peroxide to give a disulfide. The fact that more than 2 moles of hydrogen peroxide were reduced per mole of thiol indicated that the thiols were oxidized beyond the disulfide stage.

## DISCUSSION

The antithyroid activities of some thiol compounds cannot be explained by their antiperoxidase activities, as Dempsey suggested (4), because of the following observations: thiol compounds do not inhibit peroxidase, but rather are reducing agents capable of reacting as substrates for the peroxide-peroxidase system, thus competing with other substrates for the available peroxide-peroxidase complex; they are able to reduce the colored dyes formed from *p*-aminobenzoic acid and benzidine and thus show an apparent

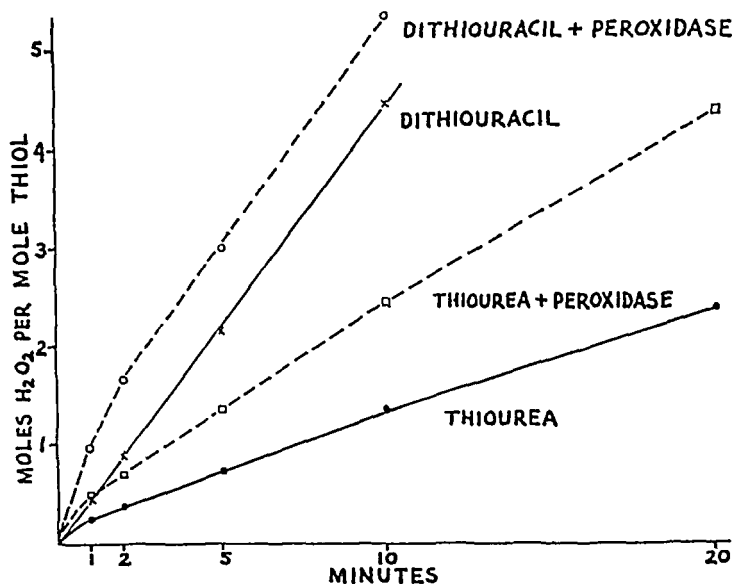


FIG. 2. Reaction of thiol compounds with hydrogen peroxide and peroxidase. The moles of hydrogen peroxide used per mole of thiol compound present initially were plotted against time in minutes. The curves illustrate the rate of disappearance of hydrogen peroxide in the presence of thiourea, thiourea plus peroxidase, 2,6-dithiouracil, and 2,6-dithiouracil plus peroxidase.

inhibition of color production by the peroxidase system; they are able to reduce hydrogen peroxide and thus remove it as a reactant in the peroxidase system.

If it could be demonstrated that hydrogen peroxide, arising from oxidase systems in the cell, is involved in thyroxine synthesis (2, 3), then an explanation of the antithyroid activities of thiol compounds might be that they reduce the hydrogen peroxide as it is formed in the thyroid cell and thus remove it from the reacting system. The reducing power would then be an important chemical characteristic of antithyroid agents.



## SUMMARY

Thiol compounds do not inhibit peroxidase but, on the contrary, are substrates for the peroxidase-hydrogen peroxide system.

Thiol compounds are oxidized by hydrogen peroxide and the rate is accelerated by peroxidase.

The author is indebted to Dr. E. J. de Beer and Dr. G. H. Hitchings for discussions of this work.

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# THE DISSOCIATION CONSTANTS OF ORGANIC CALCIUM COMPLEXES

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Cations of the alkali earth group have strong tendencies to form partially dissociated complexes in solution with the anions of carboxylic acids and other weak acids. The affinity of this association tends to increase with the number of carboxylic acid groups of the acid anion. In a considerable number of cases the effect has been studied quantitatively and expressed in terms of well defined dissociation or association constants. The interaction of calcium ions and strontium ions with citrate ions has been studied by means of the frog heart preparation, and the equilibrium expressed according to the mass action law (6). The equilibrium constants for the interaction of alkali earths and other bivalent cations with a number of carboxylic acids have been determined by the effects of the cations on the pH titration curves of the acids (2). This method has been applied also to the study of solutions containing carbonates and phosphates (3, 9).

Few of the results reported in the literature have been checked by independent methods of determining the equilibria. For this reason, it is of interest to study the interaction of calcium, barium, and strontium ions with weak organic acids by means of electrodes of the third kind. In an earlier paper (7) the interactions of the alkali earth chlorides with glycine, alanine, and egg albumin were studied by this method. In this paper, it is applied to carboxylic acids with one, two, or three carboxyl groups per molecule.

## Method

The amalgam electrode of the third kind,  $\text{PbHg}|\text{PbC}_2\text{O}_4, \text{CaC}_2\text{O}_4|\text{Ca}^{++}$ , has been previously described, as have the corresponding barium and strontium electrodes (7). Lead amalgam is in contact with a mixture of solid lead oxalate and alkali earth oxalate, the lead ion potential at the electrode depending on the potential of the alkali earth ion in the solution. The technique employed earlier in studying mixtures of amino acids and alkali earth chlorides has been applied, with one important modification, to the organic acid solutions. While it is possible to study the effect in a cell

\* Part of the expenses of this investigation was borne by a grant from The Nutrition Research Laboratories.

without liquid junction with silver-silver chloride electrodes, it has proved rather more convenient to work with the amalgam electrode as a half cell with a saturated KCl liquid junction against a silver-silver chloride electrode in saturated KCl. Consequently, the results are expressed in terms of  $p\text{Ca}$ ,  $p\text{Ba}$ , or  $p\text{Sr}$ , rather than in terms of the activities of the corresponding chlorides. The observed electromotive force contains a liquid junction potential of the order of magnitude of that occurring in pH determinations with the hydrogen or glass electrode. Cannan and Kibrick (2) have found the liquid junction potential to be negligible in the potentiometric measurements in systems containing alkali earths and organic acids.

On the assumption of constant liquid junction potentials,  $E$ , the difference of potential of the two half cells is given by the relation

$$E = E^{\circ} - \frac{RT}{2F} \ln A_{\text{Ca}} \quad (1)$$

where  $E^{\circ}$  is the standard difference of potential between the half cells,  $A_{\text{Ca}}$  is the calcium ion activity, and  $R$ ,  $T$ , and  $F$  have their usual significance.

When  $p\text{Ca}$  is defined as the negative logarithm of  $A_{\text{Ca}}$ ,

$$E = E^{\circ} + \frac{2.303RT}{2F} p\text{Ca} \quad (2)$$

or at 25°

$$E = E^{\circ} + 0.02957 p\text{Ca} \quad (3)$$

Corresponding equations hold for the barium and strontium electrodes.

#### EXPERIMENTAL

##### *Reagents—*

Calcium chloride and barium chloride were recrystallized c.p. reagents. Strontium chloride was prepared from c.p. strontium bromide by precipitation of the carbonate. After thorough washing, the precipitate was dissolved with a calculated quantity of standard 0.1 N HCl.

Potassium citrate, sodium benzoate, sodium salicylate, and racemic potassium tartrate were recrystallized c.p. reagents.

Sodium succinate, sodium lactate, and sodium acetate were prepared by neutralization of the c.p. acids with standard NaOH.

Secondary potassium *o*-phthalate was prepared by neutralization of c.p. potassium acid phthalate with KOH.

*Effect of NaCl*—Before determining the effects of the organic anions on  $p\text{Ca}$ , determinations were carried out in mixtures of  $\text{CaCl}_2$  and NaCl at three different calcium concentrations in the physiological range and at NaCl concentrations up to 0.3 molal. In these systems the electrode

reached equilibrium in a period varying from 5 minutes to 1 hour, depending on the nature of the initial and final states. Attainment of equilibrium was rapid when the two solutions being compared were of similar composition with respect to both components, and slow when there was considerable difference. After attainment there was no tendency for the E.M.F. to fluctuate by more than about  $\pm 0.3$  millivolt over a period of several hours. The equilibria obtained with any electrode were reversible. When the

TABLE I  
*pCa of NaCl-CaCl<sub>2</sub> Mixtures at 25°*

CaCl <sub>2</sub>	NaCl molality	$\mu$ , ionic strength	$\Delta E$	pCa
mm			solt	
1.25	0.00	0.00375	0.0000	3.01
	0.01	0.01375	0.0027	3.10
	0.02	0.02375	0.0048	3.17
	0.05	0.05375	0.0080	3.28
	0.10	0.10375	0.0107	3.37
	0.15	0.15375	0.0128	3.44
	0.20	0.20375	0.0152	3.52
	0.30	0.30375	0.0173	3.61
2.50	0.00	0.0075	0.0000	2.76
	0.01	0.0175	0.0024	2.84
	0.02	0.0275	0.0037	2.89
	0.05	0.0575	0.0065	2.98
	0.10	0.1075	0.0099	3.10
	0.15	0.1575	0.0118	3.16
	0.20	0.2075	0.0140	3.23
	0.30	0.3075	0.0155	3.28
3.75	0.00	0.01125	0.0000	2.61
	0.01	0.02125	0.0022	2.68
	0.02	0.03125	0.0033	2.72
	0.05	0.06125	0.0060	2.82
	0.10	0.11125	0.0097	2.94
	0.15	0.16125	0.0118	3.01
	0.20	0.21125	0.0130	3.05
	0.30	0.31125	0.0142	3.09

electrode was placed in any of the reference solutions, its potential returned to its reference value within the normal time interval.

From the electromotive force values, pCa in the salt mixtures has been computed and the results entered in Table I. The assignment of any pCa value in any series depends on a knowledge of the activity coefficient of the calcium ion in one of the reference solutions. These values have been obtained in the following manner. At three different CaCl<sub>2</sub> concentrations, 1.25, 2.50, and 3.75 millimolal, the experimental data have been

fitted by the formula

$$pCa = -\log [Ca^{++}] + 2\sqrt{\mu} - 1.40\mu \quad (4)$$

where  $[Ca^{++}]$  is the calcium ion molality and  $\mu$  is the ionic strength. This is a modification of the Debye-Hückel equation in a form developed by Bronsted and LaMer (1).

Equation 4 leads to the following values of  $pCa$  for the three reference solutions, 0.00125, 0.00250, and 0.00375 M  $CaCl_2$ : 3.01, 2.76, and 2.61, respectively. These are the standard values upon which the other values of each series are based. The value  $\Delta E$  represents the increment of E.M.F.

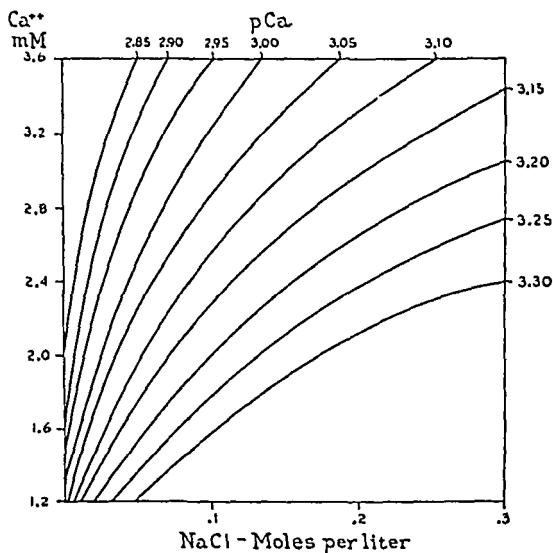


FIG. 1. The relation between  $pCa$ , calcium ion concentration, and  $NaCl$  concentration in  $NaCl-CaCl_2$  mixtures.

produced by adding a given amount of  $NaCl$  to one of the reference solutions. At  $25^\circ$ , it is related to  $\Delta pCa$  by the relation

$$\Delta pCa = 33.82\Delta E \quad (5)$$

The results are presented graphically in Fig. 1, which represents  $pCa$  as a function of  $CaCl_2$  and  $NaCl$  concentration. The function has been computed by the use of Equation 4. The results indicate that the effects are explicable on the basis of the interionic force theory. Similar results were obtained with various salt mixtures when cells without liquid junction were studied (7).

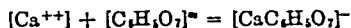
*Effects of Organic Acids*—The method followed in the case of each organic acid was to vary the concentration of acid anions at constant total calcium

concentration and constant ionic strength. The ionic strength was maintained at 0.15 by varying the amount of NaCl in the system. As a first approximation, it is assumed that variations of  $pCa$  at constant ionic strength are determined by variations of calcium ion concentration due to complex formation with the acid ion. The mechanisms postulated correspond to those assumed by Cannan and Kibrick (2) and by Hastings, McLean, Eichelberger, Hall, and Da Costa (6). In most cases the observed effects are of a larger order of magnitude than the effects to be predicted from the influence of the ionic atmosphere on the electrostatic part of the chemical potentials of ions at constant ionic strength. Undoubtedly, the electrostatic effects of specific ions become relatively more significant when the affinity of association of the complex ions is small. The validity of the treatment is greatest in those cases in which the affinity of complex formation is greatest.

*Citric Acid*—Table II contains the results of determinations of  $pCa$ ,  $pBa$ , and  $pSr$  in systems containing potassium citrate and the various alkali earth chlorides. The calcium ion concentration is calculated from the relation

$$-\Delta \log [Ca^{++}] = \Delta pCa \quad (6)$$

where the increment  $\Delta pCa$  denotes the change of  $pCa$  produced by a given amount of potassium citrate added to the citrate-free reference solution at constant total calcium concentration and ionic strength. From the value of  $[Ca^{++}]$  so obtained the apparent equilibrium constant,  $K'$ , for the complex formation is computed on the assumption that the inactivated calcium is combined reversibly and stoichiometrically with citrate to form the complex anion  $[CaC_6H_5O_7]^-$ . Thus



This is the mechanism shown to describe the equilibrium by Hastings *et al.* (6). By means of the experimental values of  $[Ca^{++}]$ ,  $K'$  is computed from the equation

$$\frac{\text{Total Ca} - [Ca^{++}]}{[Ca^{++}][\text{total citrate minus total Ca} + [Ca^{++}]]} = K' \quad (7)$$

Similar mechanisms are postulated for the barium and strontium systems and identical methods of calculation are applied.

*Dicarboxylic Acids*—The results obtained with sodium succinate and racemic potassium tartrate are presented in Table III. The ionic strength was maintained at the constant value 0.15 by adjusting the NaCl concentration to compensate for the added salt. The equilibrium constant  $K'$  is obtained from the mass law expressions for the postulated equilibria

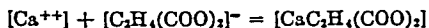


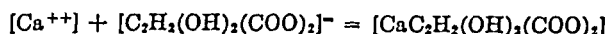
TABLE II

*Interaction of Alkali Earth Cations with Citrate Ions at 25°*

Ionic strength = 0.15.

CaCl <sub>2</sub>	K <sub>2</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub>	NaCl	ΔE	ΔpCa	[Ca <sup>++</sup> ]	Log K'
<i>mole per l.</i>	<i>mole per l.</i>	<i>mole per l.</i>	<i>volt</i>		<i>mole per l.</i>	
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
	0.0010	0.1365	0.0047	0.159	0.00173	3.29
	0.0015	0.1335	0.0072	0.244	0.00143	3.24
	0.0025	0.1275	0.0116	0.392	0.00101	3.16
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
	0.0005	0.1320	0.0011	0.037	0.00459	3.00
	0.0010	0.1290	0.0024	0.081	0.00415	3.13
	0.0025	0.1200	0.0070	0.237	0.00290	3.26
	0.0050	0.1050	0.0138	0.469	0.00170	3.10
Mean.....						3.17
BaCl <sub>2</sub>				ΔpBa	[Ba <sup>++</sup> ]	
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
	0.0005	0.1395	0.0020	0.068	0.00214	3.08
	0.0010	0.1365	0.0038	0.129	0.00186	2.98
	0.0015	0.1335	0.0062	0.210	0.00154	3.06
	0.0025	0.1275	0.0092	0.312	0.00122	2.93
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
	0.0025	0.1200	0.0060	0.203	0.00313	2.98
	0.0050	0.1050	0.0117	0.398	0.00200	2.88
Mean.....						2.98
SrCl <sub>2</sub>				ΔpSr	[Sr <sup>++</sup> ]	
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
	0.0010	0.1365	0.0035	0.113	0.00193	2.84
	0.0015	0.1335	0.0060	0.203	0.00156	3.02
	0.0025	0.1275	0.0096	0.325	0.00118	2.98
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
	0.0010	0.1290	0.0020	0.068	0.00428	2.78
	0.0025	0.1200	0.0060	0.203	0.00313	2.98
	0.0050	0.1050	0.0126	0.428	0.00187	2.95
Mean.....						2.92

and



These are the equilibria postulated by Cannan and Kibrick (2) for dicarboxylic acids.



TABLE III

*Interaction of Alkali Earth Cations with Succinate and Racemic Tartrate at 25°*  
 Ionic strength = 0.15.

$\text{CaCl}_2$	$\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$	$\text{NaCl}$	$\Delta E$	$\Delta p\text{Ca}$	$[\text{Ca}^{++}]$	$\text{Log } K'$
mole per l.	mole per l.	mole per l.	volt		mole per l.	
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
	0.0050	0.1275	0.0011	0.037	0.00230	1.25
	0.0100	0.1125	0.0024	0.081	0.00207	1.34
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
	0.0050	0.1200	0.0007	0.024	0.00473	1.08
	0.0100	0.1050	0.0013	0.044	0.00452	1.05
	0.0200	0.0075	0.0026	0.088	0.00403	1.07
Mean .....						1.16 ( $\pm 0.12$ )
$\text{BaCl}_2$				$\Delta p\text{Ba}$	$[\text{Ba}^{++}]$	
0.0050	0.0000	0.1350	0.0000	0.000	0.0050	
	0.0050	0.1200	0.0050	0.017	0.00481	0.91
	0.0100	0.1050	0.0013	0.044	0.00452	1.05
	0.0200	0.0075	0.0023	0.068	0.00428	0.94
Mean .....						0.97 ( $\pm 0.06$ )
	$\text{K}_2(\text{C}_4\text{H}_4\text{O}_6)$					
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
	0.0040	0.1305	0.0038	0.128	0.00186	2.01
	0.0060	0.1245	0.0050	0.169	0.00170	1.96
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
	0.0040	0.1230	0.0030	0.101	0.00396	1.95
	0.0060	0.1170	0.0040	0.135	0.00366	1.90
Mean .....						1.95 ( $\pm 0.04$ )
$\text{SrCl}_2$				$\Delta p\text{Sr}$	$[\text{Sr}^{++}]$	
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
	0.0040	0.1305	0.0035	0.118	0.00191	1.96
	0.0060	0.1245	0.0046	0.156	0.00175	1.91
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
	0.0040	0.1230	0.0032	0.108	0.00390	1.99
	0.0060	0.1170	0.0040	0.135	0.00366	1.90
Mean .....						1.94 ( $\pm 0.04$ )

In the case of the calcium tartrate systems, irreversible potentials were encountered, the apparent effect of the tartrate on  $p\text{Ca}$  being considerably greater than the effect estimated from Cannan and Kibrick's results. The

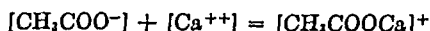
anomalous results were judged to be caused by the formation of insoluble lead tartrate at the electrode.<sup>1</sup> When the tartrate concentration was less than 0.01 molal, this difficulty was avoided with the strontium and barium electrodes because of the much lower lead ion concentrations at the electrode. This is a result of the greater solubilities of the strontium and barium oxalates compared with calcium oxalate, the lead ion concentration varying inversely as the solubility of the alkali earth oxalate.

TABLE IV

*Interaction of Sodium Acetate and Sodium Lactate with Calcium Ions at 25°*  
Ionic strength = 0.15.

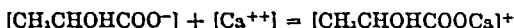
CaCl <sub>2</sub>	Na(C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> )	NaCl	ΔE	ΔpCa	[Ca <sup>++</sup> ]	Log K'
<i>mole per l.</i>	<i>mole per l.</i>	<i>mole per l.</i>	<i>volt</i>		<i>mole per l.</i>	
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
0.0025	0.0100	0.1325	0.0003	0.010	0.00244	0.40
0.0025	0.0200	0.1225	0.0009	0.030	0.00233	0.54
0.0025	0.0300	0.1125	0.0014	0.054	0.00211	0.64
Mean .....						0.53 (±0.10)
	Na(C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> )					
0.0025	0.0000	0.1425	0.0000	0.000	0.00250	
0.0025	0.0050	0.1375	0.0007	0.024	0.00237	1.05
0.0025	0.0100	0.1325	0.0010	0.034	0.00231	0.93
0.0025	0.0200	0.1225	0.0015	0.051	0.00222	0.82
0.0050	0.0000	0.1350	0.0000	0.000	0.00500	
0.0050	0.0100	0.1250	0.0006	0.020	0.00478	0.68
0.0050	0.0200	0.1150	0.0010	0.034	0.00462	0.62
Mean .....						0.82 (±0.16)

*Monocarboxylic Acids*—The interaction of sodium acetate and sodium lactate with calcium ions was studied at an ionic strength of 0.15. The results are given in Table IV. The apparent constants  $K'$  are calculated for the reversible equilibria



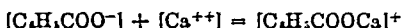
<sup>1</sup> The formation of *soluble* partially dissociated organic lead complexes theoretically leads to no serious error unless the dissociation constant is of a much lower order of magnitude than that of the calcium complex. The lead ion concentration is still determined by calcium ions and the solubility products of the oxalates. However, a very small percentage of the combined anions should be regarded as bound to lead rather than to calcium, the ratio of lead ions to calcium ions being of the order of 1:100.

and

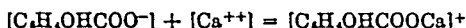


These are the complexes assumed by Cannan and Kibrick (2).

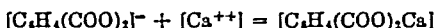
*Aromatic Carboxylic Acids*—Three aromatic carboxylic acids were studied with calcium chloride and barium chloride respectively. Sodium benzoate and sodium salicylate showed very small effects with both calcium and barium, leading to values of  $\log K'$  that were negative in all systems except calcium salicylate, in which it had a very small positive value. The values of  $K'$  were calculated for the assumed equilibria



and



The values of  $K'$  were of practically the same magnitude for the salicylates and benzoates. Potassium *o*-phthalate was also studied. The apparent equilibrium constant  $K'$  was calculated for the association



For the dicarboxylic aromatic acid  $\log K'$  had a positive value only slightly less than that of the corresponding succinate complexes. The results for all three aromatic acids are included in Table V.

*Bicarbonate Systems*—An attempt was made to determine the interaction of calcium and barium ions with bicarbonate ions in the physiological range of concentrations (0.0025 *M*  $\text{CaCl}_2$ , 0.025 *M*  $\text{NaHCO}_3$ ,  $\text{NaCl}$  to ionic strength 0.15). In all such attempts irreversible potentials were encountered which seemed to be of far too high an order of magnitude to represent the effect on  $p\text{Ca}$  or  $p\text{Ba}$ . The effect on the electrode potential was approximately 1 millivolt per *mM* of bicarbonate ion per liter, which would lead to a value of the order of 2 for  $\log K'$ , assuming the equilibrium



The results of Greenwald (3) based on pH titrations lead to a value of about 0.8 for this constant.<sup>2</sup> The large irreversible potentials obtained with the electrode of the third kind appear to be produced by the formation of insoluble lead bicarbonate at the electrode. This effect would clearly vitiate results obtained with this particular electrode in biological fluids containing bicarbonate ions. A satisfactory electrode should probably be one whose metallic cation forms a soluble bicarbonate.

<sup>2</sup> In an exact formulation of this equilibrium the formation of undissociated  $[\text{CaCO}_3]$  should be taken into account. As Greenwald (3) has shown, both complexes are formed in the physiological range of pH. Because of the formation of insoluble lead salts, neither equilibrium can be studied with the lead amalgam electrode.

TABLE V

*Interaction of Aromatic Acids with Calcium and Barium Ions at 25°*  
 Ionic strength = 0.15.

Sodium benzoate							
CaCl <sub>2</sub>	C <sub>6</sub> H <sub>5</sub> COONa	NaCl	ΔE	ΔpCa	[Ca <sup>++</sup> ]	Log K'	Mean log K'
<i>mole per l.</i>	<i>mole per l.</i>	<i>mole per l.</i>	<i>volt</i>		<i>mole per l.</i>		
0.0025	0.000	0.1425	0.0000	0.000	0.00250		
	0.025	0.1175	0.0002	0.007	0.00246	-0.18	
	0.050	0.0925	0.0002	0.007	0.00246	-0.48	
0.0050	0.000	0.1350	0.0000	0.000	0.00500		
	0.025	0.1100	0.0004	0.014	0.00484	-0.18	
	0.050	0.0850	0.0005	0.017	0.00481	-0.12	-0.24
BaCl <sub>2</sub>				ΔpBa	[Ba <sup>++</sup> ]		
0.0025	0.000	0.1425	0.0000	0.000	0.00250		
	0.025	0.1175	0.0000	0.000	0.00250	<0	
	0.050	0.0925	0.0002	0.007	0.00246	-0.48	
0.0050	0.000	0.1350	0.0000	0.000	0.00500		
	0.050	0.0850	-0.0006	-0.020	(0.00524)	<0	<0
Sodium salicylate							
CaCl <sub>2</sub>	C <sub>6</sub> H <sub>4</sub> (OH)COONa	NaCl	ΔE	ΔpCa	[Ca <sup>++</sup> ]	Log K'	Mean log K'
<i>mole per l.</i>	<i>mole per l.</i>	<i>mole per l.</i>	<i>volt</i>		<i>mole per l.</i>		
0.0025	0.000	0.1425	0.0000	0.000	0.00250		
	0.025	0.1175	0.0005	0.017	0.00240	0.22	
	0.050	0.0925	0.0007	0.024	0.00237	0.04	
0.0050	0.000	0.1350	0.0000	0.000	0.00500		
	0.025	0.1100	0.0006	0.020	0.00478	0.25	
	0.050	0.0850	0.0007	0.024	0.00473	0.06	0.14
BaCl <sub>2</sub>				ΔpBa	[Ba <sup>++</sup> ]		
0.0025	0.000	0.1425	0.0000	0.000	0.00250		
	0.025	0.1175	0.0003	0.010	0.00245	-0.10	
	0.050	0.0925	0.0003	0.010	0.00245	-0.40	-0.25

The figure in parentheses is an apparent value. The real value cannot be greater than 0.005.

*Cells without Liquid Junction*—A number of determinations have been made with the lead amalgam electrode and a silver chloride electrode in a cell without liquid junction, as in the earlier studies on amino acids (7).

The measurements have been made in the barium chloride-potassium citrate system in which relatively large effects are known to occur.

The electromotive force,  $E$ , at  $25^\circ$  is given by the relation

$$E = E^\circ - 0.0837 \log A_{\text{BaCl}_2} \quad (8)$$

or

$$E = E^\circ - 0.02957 \log A_{\text{Ba}} - 0.05915 \log A_{\text{Cl}} \quad (9)$$

where

$$A_{\text{BaCl}_2} = \sqrt{A_{\text{Ba}} A_{\text{Cl}}^2} \quad (10)$$

TABLE VI

*Interaction of BaCl<sub>2</sub> and Potassium Citrate from Electromotive Force of Cells without Liquid Junction*

Ionic strength 0.15, temperature  $25^\circ$ .

BaCl <sub>2</sub>	K <sub>2</sub> C <sub>6</sub> H <sub>5</sub> O	NaCl	$\Delta E$	$-0.05915 \Delta \log \text{Cl}^-$	$\Delta p_{\text{Ba}}$	$\log K'$
<i>mole per l.</i>	<i>mole per l.</i>	<i>mole per l.</i>	<i>volt</i>	<i>volt</i>		
0.0025	0.0000	0.1425	0.0000	0.0000	0.000	
	0.0010	0.1365	0.0052	0.0011	0.139	3.07
	0.0015	0.1335	0.0082	0.0016	0.223	3.12
	0.0025	0.1275	0.0125	0.0028	0.328	3.00
0.0050	0.0000	0.1350	0.0000	0.0000	0.000	
	0.0025	0.1200	0.0088	0.0028	0.203	2.98
	0.0050	0.1050	0.0183	0.0059	0.419	2.92
Average .....						3.02

The activities  $A_{\text{BaCl}_2}$ ,  $A_{\text{Ba}}$ , and  $A_{\text{Cl}}$  refer respectively to the electrolyte, the cation, and the anion. In interpreting the results, it is assumed that the activity coefficient of the chloride ion is constant at constant ionic strength. Then

$$E = E^\circ + 0.02957 p_{\text{Ba}} - 0.05915 \log M_{\text{Cl}} \quad (11)$$

From the data, values of  $p_{\text{Ba}}$  are obtained. The results imply no assumptions as to the liquid junction potential, but depend on the assumption regarding the chloride ionic activity coefficient. The modification of the method is analogous to that of Harned's use of cells without liquid junction to determine the dissociation constants of weak acids and bases (4, 5).

Values of  $\log K'$  are calculated as before from the values of  $p_{\text{Ba}}$ . They agree quite well with those obtained in the series of measurements with liquid junction. The results are included in Table VI.

## DISCUSSION

The results obtained by electrometric determinations of  $pCa$ ,  $pBa$ , and  $pSr$  in mixtures of the alkali earths and organic acids are conveniently summarized by tabulating the values of  $\log K'$ . In every case it is assumed that the complex formation can be expressed as a reversible bimolecular reaction of the type

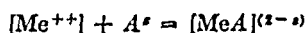


TABLE VII  
*Equilibrium Constants of Complex Formation*

$$[Me^{++}] + [A^z] = [MeA^{2-z}]; K' = \frac{[MeA]}{[Me^{++}][A]}$$

Ionic strength 0.15.

	Values of $\log K'$		
	Ca	Sr	Ba
Citrate .....	3.17 (3.22)*	2.92 (2.70)*	2.98
Racemic tartrate .....	(1.80)†	1.94 (1.65)†	1.95 (1.62)†
Succinate .....	1.16 (1.20)†	(1.06)†	0.97 (1.03)†
<i>o</i> -Phthalate .....	1.07		0.92
Lactate .....	0.82 (1.07)†	(0.70)†	(0.55)†
Acetate .....	0.53 (0.53)†	(0.43)†	(0.39)†
Salicylate .....	0.14		<0
Benzoate .....	<0		<0

Values for a number of these constants have been given by Topp and Davies (10) and by McDougall and Larson (8). Those of the former are based on conductivities, the latter on solubilities. In general the agreement is satisfactory.

\* Hastings *et al.* (6). Ionic strength, 0.155 to 0.165.

† Cannan and Kibrick (2). Ionic strength, about 0.2.

where  $z$  is the number of negative charges of the acid anion, corresponding to the number of charged carboxyl groups.  $[Me^{++}]$  denotes the metallic cation. The charge of the complex, represented by the quantity  $(2 - z)$ , may be positive, negative, or zero. The values obtained from Tables II to V are summarized in Table VII and compared with those obtained by independent methods.

In most cases there appears to be no serious discrepancy in any of the results obtained from the three independent methods, electrometric calcium, electrometric pH, and the frog heart preparation. In all cases in which the magnitude of the effect is great enough to be significant the interaction of the alkali earth cations with organic anions can accordingly be represented as a bimolecular reversible combination to form a univalent

cation, an uncharged molecule, or a univalent anion, depending on the anionic charge. The affinity of this association, as measured by  $K'$ , the equilibrium constant, increases with the number of carboxyl and hydroxyl groups of the anion. It is greater for aliphatic acids than for benzoic acid and its derivatives, especially in the case of monocarboxylic acids.

#### SUMMARY

1. The interaction of calcium, barium, and strontium ions with the anions of weak organic acids has been studied with electrodes of the third kind. The acids studied include citric, tartaric, succinic, lactic, phthalic, benzoic, and salicylic.

2. The effects are formulated in terms of a bimolecular combination of cation and anion to form a univalent complex cation or anion, or an uncharged molecule.

3. From the results the equilibrium constants of the organic metal complex are calculated, and compared with the values obtained from pH titrations and from the frog heart preparation. No serious disagreement was found in the series of results reported.

4. The tendency to complex formation was greatest for the tricarboxylic acid, and least for monocarboxylic acids. It is less for aromatic acids than for aliphatic and greater for hydroxy acids than for the unsubstituted homologues.

5. The electrode was found to yield erroneous results in bicarbonate systems. The implications of this error for determinations in biological fluids were pointed out.

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# FACTORS AFFECTING THE ENZYMIC DESTRUCTION OF CAROTENE IN ALFALFA\*

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In a previous paper on the enzymic nature of the carotene-destroying system of alfalfa, Mitchell and Hauge (1) presented evidence which indicates that the carotene-destroying enzyme in alfalfa is a lipoxidase. The following experiments are a continuation of this work and show the effects of various factors upon the activity of the enzyme in alfalfa.

## EXPERIMENTAL

In these experiments the term "whole alfalfa" is used to indicate the part of the plant which is harvested in the usual hay-making operation. In all cases in which defrosted tissue was used the material was frozen at  $-15^{\circ}$  and defrosted immediately before use. In part of the experiments, only the leaves were subjected to treatment, while in others whole alfalfa was used. In all experiments, only the leaves were used for carotene determinations. The carotene content of the fresh leaves was also determined in each experiment. All samples were blanched with steam before carotene determinations were made. Carotene was determined by a modification (2) of the method of Moore and Ely (3). Measurements were made with a Beckman spectrophotometer at 4360 A.

*Effect of Modification of Cell Structure*—In this experiment, 5 gm. samples of alfalfa leaves were divided into three series. Each series was incubated at  $37^{\circ}$ .

*Series 1*—The leaf structure was preserved as nearly as possible in its initial condition by incubating fresh leaves over water in desiccators. By this procedure the leaves were held in an atmosphere saturated with water vapor, which greatly retarded wilting and prevented desiccation.

*Series 2*—Fresh leaves were permitted to wilt and to desiccate by incubating in open beakers.

*Series 3*—Defrosted leaves, in which cell permeability had been increased by freezing (4), were incubated in beakers which were covered with watch-glasses to retard the loss of moisture.

Samples of each series were removed at intervals up to 24 hours and were analyzed for carotene. The results are presented in Fig. 1.

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The fresh leaves which were incubated in an atmosphere saturated with water vapor (Series 1) showed but little evidence of wilting even after 24 hours and lost carotene very slowly.

In the fresh leaves, which were permitted to wilt and become dry (Series 2), carotene destruction was negligible in the first 4 or 5 hours. During this period little wilting was noted. After 8 hours, the leaves were definitely wilted and the destruction of carotene was appreciable. After 24 hours the leaves were quite dry and had lost approximately twice as much carotene as the leaves in Series 1.

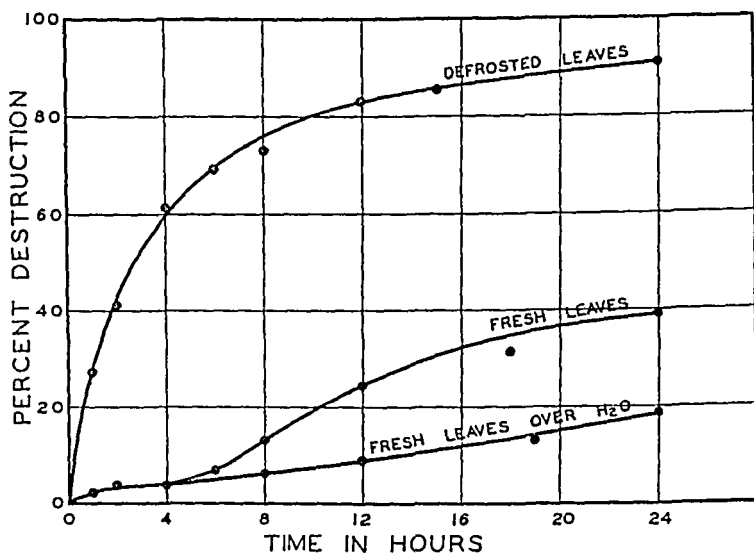


FIG. 1. Loss of carotene during the incubation of fresh leaves, fresh leaves over water, and defrosted leaves.

The turgidity of the fresh leaves had a marked inhibitory effect on the activity of the enzyme. That the water content is not directly responsible for this inhibition is indicated by the fact that defrosted leaves (Series 3) lost carotene very rapidly in spite of a high retention of water. In fresh leaves the enzyme was apparently unable to come into contact with the substrate as long as the leaves remained turgid. However, when the cell membranes were ruptured or otherwise modified by freezing, rapid loss of carotene occurred. This suggests that cell permeability limits carotene destruction. It seems logical to conclude that little loss of carotene will occur under field conditions until wilting takes place.

*Effect of Soil Fertility*—To study the effect of soil fertility on enzymic destruction, plants from two series of fertility plots were studied. In Series A, the untreated plot had not had a fertilizer application for 23 years.

The average annual yield of hay was 4527 pounds per acre. The treated plot received manure and 400 pounds of 2-12-6 fertilizer per acre in a 4 year rotation of corn, soy bean, wheat, and alfalfa. The average annual yield of hay was 5950 pounds per acre. In Series B, the plots had been growing alfalfa continuously for 28 years. The untreated plot had had no fertilizer applications and yielded an average of 6388 pounds of hay per acre per year. The treated plot had received 80 pounds of potash and superphosphate at the rate of 80 pounds of  $P_2O_5$  per acre every 2 years. Ground limestone had been added at intervals to the fertilized plot. The average yield of hay from this plot was 7791 pounds per acre per year.

Samples of alfalfa were obtained when the plants were about 8 inches high. The leaves were removed from the stems and were frozen. The frozen leaves were defrosted and incubated in closed Petri dishes for 3 hours at 37°.

TABLE I  
*Effect of Soil Fertility on Carotene-Destroying Activity of Alfalfa Leaves*

Series	Fertilization	Carotene		Loss per cent
		Fresh γ per gm. dry weight	Defrosted, incubated γ per gm. dry weight	
A	None	504	235	53.4
	Manure, N, P, K	557	232	58.3
B	None	480	196	59.2
	Lime, P, K	492	206	58.1

Results (Table I) show that soil fertility had no effect on the carotene-destroying activity of alfalfa leaves.

*Effect of Stage of Growth*—Alfalfa is usually cut for hay when in early bloom because the protein and carotene contents rapidly decrease after this point is reached (5). The possibility that there may be changes also in the carotene-destroying activity was investigated by measuring the enzyme activity at various stages of growth.

Samples of whole alfalfa were taken after the second cutting at intervals up to the time of the third cutting. Each sample was divided into two parts. One portion was incubated at 37° for 24 hours, after which the leaves were removed for analysis. The leaves of the other portion were removed from the stems and were frozen. The leaves were defrosted and incubated at 37° for 3 hours.

The data, presented in Table II, indicate a slight decrease in enzymic activity with increasing age of the plants up to very early bloom stage. Unfortunately the entire field was harvested at this stage, so that no later

samples were available to show the effect of more advanced stages of maturity.

*Effect of Variety*—Since genetic constitution may greatly affect the chemical composition of plants, four varieties of alfalfa were used to investigate the possibility of differences in carotene-destroying activity.

In the first experiment, plots of Buffalo, Ranger, Grimm, and Turkestan alfalfa were harvested when in the early bloom stage of the second growth. Fresh leaves of each variety were incubated in open Petri dishes for 24 hours at 37° and defrosted leaves were incubated in closed Petri dishes for 3 hours. The carotene determinations are shown in Table III.

In the second experiment the alfalfa plots were harvested during the early bloom stage of the third growth. Defrosted leaves of each variety were treated as in the first experiment. The fresh material, however, was

TABLE II  
*Effect of Stage of Growth on Carotene-Destroying Activity of Alfalfa Leaves*

Date of harvest	Height of plants	Carotene			Loss	
		Fresh	Fresh, incubated	Defrosted, incubated	Fresh, incubated	Defrosted, incubated
	in.	$\gamma$ per gm. dry weight	$\gamma$ per gm. dry weight	$\gamma$ per gm. dry weight	per cent	per cent
July 26.....	8-10	540		166		69.3
Aug. 2.....	10-12	510	238	179	53.3	64.9
" 10.....	12-14	439	199	181	54.7	58.8
" 20.....	14-16*	398	217	181	45.5	54.5

\* A few blossoms.

treated in a different manner in order to duplicate as closely as possible the conditions that would prevail in the field on a typical hay-curing day. The whole alfalfa was dried in a Despatch oven with circulating air at 30-35° for 24 hours. This temperature range approximates that which was found about 2 inches above alfalfa curing in the field on a good hay-curing day. The results of the experiment are presented in Table III.

No consistent differences were obtained in the enzyme activity of the four varieties. If the varieties are arranged in order of increasing loss of carotene, the order for one method of treatment may not be the same as that for the other methods. This may be due to factors other than enzyme content which operate to different extents under the various conditions. The incubation of defrosted leaves should be the more accurate method of estimating the actual enzyme content, since such factors as cell permeability and rate of desiccation are eliminated.

Although the four varieties of alfalfa which were tested did not differ

consistently in carotene-destroying activity, it should not be assumed that varieties of low activity do not exist or cannot be produced by altering the genetic constitution of present varieties. The development of such varieties would minimize the loss due to enzyme activity during field curing. Consequently, alfalfa of higher carotene content might be produced by the curing processes now in use.

*Relative Effect of Sunlight and Enzyme Activity on Loss of Carotene in Alfalfa*—The investigations of Guilbert (6) and of Jones *et al.* (7) on the destructive effect of sunlight on the carotene in alfalfa were conducted at temperatures considerably higher than those which prevail under field

TABLE III

*Effect of Variety on Carotene-Destroying Activity of Second and Third Cutting Alfalfa Leaves*

Variety	Carotene			Loss	
	Fresh	Fresh, incubated	Defrosted, incubated	Fresh, incubated	Defrosted, incubated
2nd cutting					
	<i>γ per gm. dry weight</i>	<i>γ per gm. dry weight</i>	<i>γ per gm. dry weight</i>	<i>per cent</i>	<i>per cent</i>
Buffalo.....	388	234	167	39.7	57.0
Ranger.....	382	233	165	39.0	56.8
Grimm.....	365	209	178	42.7	51.2
Turkestan.....	344	204	133	40.7	61.3
3rd cutting					
Buffalo.....	449	268	274	40.3	39.0
Ranger.....	478	312	255	34.7	46.7
Grimm.....	497	343	314	31.0	36.8
Turkestan.....	486	349	229	28.2	52.9

curing conditions. Since it is conceivable that their conclusions are not valid for field curing conditions, an experiment was conducted to determine the relative effects of sunlight and of enzyme activity on carotene destruction during field curing.

Fresh whole alfalfa was spread on the ground in a thin layer at 2 p.m. and was exposed to sunlight and other field curing conditions until 4 p.m. the following day. The first afternoon was a good hay-curing day for Indiana, being a cloudless, warm day with a moderately strong wind. The second day was partly cloudy. The temperature of the air immediately above the alfalfa was measured every half-hour. Portions of the sample were removed for carotene and moisture determinations at 7 p.m., 7.30 a.m., and 4 p.m.

To measure the destruction of carotene in the absence of sunlight, a sample of the fresh whole alfalfa was treated in a Despatch oven with circulating air at temperatures which coincided as nearly as possible with those which were observed above the leaves outside. The sample was moved outdoors at 7 p.m. and was covered with paper to exclude late evening and early morning sunlight. It was returned to the oven at 7.30 a.m. the next morning. Portions were removed for analysis at 7 p.m., 7.30 a.m., and 4 p.m.

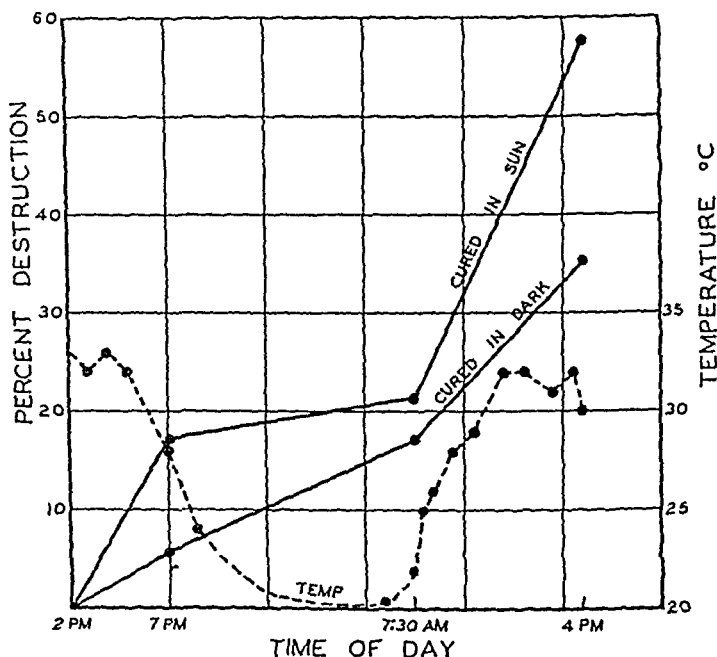


FIG. 2. Effect of light and enzyme activity on the destruction of carotene in alfalfa leaves.

The data are summarized in Fig. 2. The loss of carotene due to sunlight and enzyme action in the sun-cured alfalfa was 57.8 per cent, while the loss in the oven-cured alfalfa due to enzyme action alone was 35.2 per cent. These values do not support the conclusion of the previous investigators that enzyme action is of minor importance when compared to the destructive effect of sunlight. Climatic conditions, however, may greatly alter the relative effects of sunlight and enzyme activity. In regions where low humidity and high temperature favor rapid drying, enzymic destruction of carotene may be considerably less than was found under Indiana conditions. Unnecessary exposure to sunlight after the hay is cured may also cause further destruction of carotene.

The apparent destructive action of sunlight may be explained in a number of ways. (a) Usually the stomata of leaves are closed in the absence of light (8). By causing stomatal opening, sunlight accelerates transpiration, which results in more rapid wilting of the leaves. (b) Sunlight may produce leaf temperatures which are somewhat higher than the temperature of the surrounding air (8), with the result that enzymic destruction will be increased both by increased wilting and by the effect of temperature upon the enzyme itself. (c) In the presence of chlorophyll, carotene in solutions is known to be destroyed rapidly by sunlight (9) and it is conceivable that such destruction also occurs in the leaf.

An experiment similar to that described above was conducted in which autoclaved whole alfalfa was dried in the sun and in the dark. No loss of carotene occurred in the sample dried in the dark. However, the sample dried under field conditions in the sun lost both chlorophyll and carotene until the leaves were bleached almost white. The sample lost even more carotene than did unautoclaved sun-cured alfalfa under the combined effects of sunlight and enzyme action. This confirms the work of Jones *et al.* (7). The reason for the greater loss in autoclaved alfalfa under field conditions is obscure. Ultraviolet light is apparently not responsible, since a sample of autoclaved alfalfa leaves which was exposed at 35° to ultraviolet light from a mercury arc lamp lost only 9 per cent in 12 hours.

In view of these experiments, it appears that the estimation of the effects of sunlight and enzyme on carotene destruction cannot be accomplished by comparing autoclaved and unautoclaved alfalfa dried under field conditions. The comparison of unautoclaved alfalfa cured in the sun and unautoclaved alfalfa cured in the dark appears to be the most feasible method at the present time.

#### SUMMARY

Enzymic destruction of carotene in alfalfa leaves was retarded as long as the tissues remained turgid, but increased rapidly with wilting. Since the loss of carotene was very rapid when the cells had been ruptured or otherwise modified by freezing, it appears that cell permeability limits carotene destruction. Under field conditions, little loss of carotene occurs until wilting takes place.

Soil fertility had no significant effect on the carotene-destroying activity of alfalfa leaves.

As the plants approached maturity, there was a slight decrease in carotene-destroying activity.

There were no consistent differences in the carotene-destroying activity of the four varieties of alfalfa studied.

Enzymic destruction of carotene during field curing appeared to be greater than destruction by light.

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## TRYPTOPHANE STUDIES

### I. THE EFFECT OF NIACIN ON THE UTILIZATION OF TRYPTOPHANE\*

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Although previous work (1) has demonstrated that either tryptophane or niacin is completely and separately active in correcting the poor growth which results when rats are fed a niacin-low diet which contains corn, little other than indirect evidence has been obtained to explain the mechanism of this syndrome. Woolley has recently shown (2) that the growth-inhibiting effect of 3-acetylpyridine in mice can be counteracted by tryptophane and suggests that the "action of corn may be related to the occurrence in it of a structural analogue of nicotinic acid which competes with that vitamin just as 3-acetylpyridine does." While this hypothesis is attractive and may indeed be correct, it appears to fall short of explaining the fact that the syndrome produced with corn can be duplicated with non-corn rations by adding tryptophane-free proteins or acid-hydrolyzed proteins to niacin-low rations which contain marginal amounts of tryptophane (3).

In an effort to elucidate the relationship between niacin and tryptophane an experiment was devised to determine the effect of niacin on tryptophane utilization. This was done by determining the balance between ingested tryptophane and the tryptophane accounted for by gain in body weight plus that excreted in feces and urine.

Inasmuch as an accurate and preferably rapid method for tryptophane analysis was a prerequisite to this study, preliminary experiments were made to ascertain how tryptophane could be determined most satisfactorily in animal tissues, urine, and feces.

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the generous supply of crystalline B vitamins, and to the Abbott Laboratories, North Chicago, Illinois, for the generous supply of haliver oil.

## EXPERIMENTAL

*Determination of Tryptophane*—The problem of tryptophane analysis lies not in its measurement, since it can be rapidly and accurately determined by microbiological methods, but rather in the manifest difficulties of preparing the sample for analysis in such a manner as to liberate all of the tryptophane without its destruction. Although several methods have been proposed for the liberation of tryptophane (4-6), each is subject to certain disadvantages and limitations. The enzymatic method of Wooley *et al.* (5) probably offers the highest degree of accuracy but is cumbersome and time-consuming; therefore the alternative procedures of alkaline hydrolysis were chosen as a basis for the present study.

Greene and Black (4) proposed the use of anhydrous  $\text{Ba}(\text{OH})_2$  for the liberation of tryptophane and obtained their results by multiplying the assay values by 2 to correct for assumed complete racemization. Wooley *et al.* (5) indicated that irregular results for tryptophane were obtained when 5 N NaOH was used for hydrolysis and suggested that the extent of racemization of pure *l*(-)-tryptophane is variable. Stokes *et al.* (6) reported favorably on the use of 5 N NaOH at  $121^\circ$  for the liberation and racemization of tryptophane from proteins, but emphasized the importance of sample size and time of hydrolysis.

In order to check these observations, casein, casein plus glucose, pure *l*(-)-tryptophane alone and in recovery experiments with casein, and *l*(-)-tryptophane plus gelatin were treated with various concentrations of NaOH and  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ . The procedure used for hydrolysis with 5 N  $\text{Ba}(\text{OH})_2$  was that reported by Greene and Black (4), except that  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  was used instead of  $\text{Ba}(\text{OH})_2$  (with proper correction for the water of crystallization).

The materials treated, the method of treatment, and the analytical results obtained are compiled in Table I. Tryptophane was measured microbiologically by the omission of tryptophane and the inclusion of niacin in the medium of Krehl *et al.* (7). A representative standard curve is shown in Fig. 1.

It is evident from Table I that good analytical results were obtained for the tryptophane content of casein with either 5 N NaOH or 5 N  $\text{Ba}(\text{OH})_2$ , although the  $\text{Ba}(\text{OH})_2$  method is longer and more troublesome than NaOH treatment. Neutralization of the NaOH by HCl with the subsequent presence of NaCl in the sample should not prove troublesome for most samples because (a) *Lactobacillus arabinosus* is able to tolerate rather large concentrations of NaCl, and (b) the dilution of the sample in most cases reduces the NaCl concentration below critical levels. Concentrations of NaOH below 5 N gave less secure results, although 14 per cent  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  appears to be about as effective as 5 N  $\text{Ba}(\text{OH})_2$  in hydrolyzing casein

TABLE I

*Tryptophane Assay Results on Pure Tryptophane and Casein with Lactobacillus arabinosus*

Material treated	Method of treatment, at 121°	Tryptophane content*	Tryptophane recovery†
		per cent	per cent
Casein, † 1 gm.	20 cc. 1 N NaOH, 5 hrs.	0.66	
" 1 "	20 " 2.5 N NaOH, 7 hrs.	1.22	
" 1 "	20 " 5 N NaOH, 5 hrs.	1.23	
		(1.16-1.28)	
" 1 "	20 " 5 " " 7 "	1.28	
" 2 " + glucose, 2 gm.	40 " 5 " " 7 "	1.10	
Casein, 1 gm. + l(-)-tryptophane, 10 mg.	20 " 5 " " 7 "		106
Casein, 1 gm.	25 " 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O, 5 hrs.	1.15	
" 1 "	20 cc. 5 N Ba(OH) <sub>2</sub> , 7 hrs.	1.28	
" 1 " + l(-)-tryptophane, 10 mg.	20 " 5 " " 7 "		66
Casein, 1 gm. + l(-)-tryptophane, 10 mg.	25 " 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O		78
l(-)-Tryptophane, 10 mg.	H <sub>2</sub> O, 5 hrs.		93
" 10 "	20 cc. 2 N NaOH, 5 hrs.		94
" 10 "	20 " 5 " " 5 "		(88-92)
" 10 "	20 " 5 " " 7 "		81
" 20 "	40 " 5 " " 7 "		76
" 5 "	25 " 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O, 7 hrs.		(84-83)
" 5 "	25 cc. 5 N NaOH, 5 hrs.		(84-92)
" 10 "	20 " 5 " Ba(OH) <sub>2</sub> , 7 hrs.		(46-48)
" 5 " + gelatin, 0.5 gm.	20 " 5 " NaOH, 5 hrs.		84
l(-)-Tryptophane, 5 mg. + gelatin, 0.5 gm.	20 " 5 " Ba(OH) <sub>2</sub> , 7 hrs.		44

\* Values were obtained by multiplying the actual values by 2, assuming complete racemization.

† Values, as found by microbiological assay. The figures in parentheses show the range when two or more samples were analyzed.

‡ Labco vitamin-free casein.

under the conditions employed. The addition of glucose to casein resulted in somewhat lower tryptophane values with either reagent. Increasing the autoclaving time beyond 5 hours resulted in a poorer recovery of l(-)-tryptophane.

The assumption of complete racemization of pure l(-)-tryptophane in

the presence of strong alkali is unwarranted, particularly when 5 N NaOH is used (Table I). This fact, plus the variable results obtained with 5 N Ba(OH)<sub>2</sub> treatment of *l*(-)-tryptophane, raised the question as to whether the recoveries obtained with this reagent, which approximated 50 per cent, were actually the result of complete racemization or the fortuitous result of about 50 per cent destruction of *l*(-)-tryptophane.

Since *Lactobacillus arabinosus* responds only to the *l*(-) form of tryptophane, the question of racemization *versus* destruction could not be answered by the use of this test. The fate of tryptophane could be determined, however, by applying a chemical test to the alkali-treated tryptophane solutions, inasmuch as no chemical differentiation of the enantiomorphs of tryptophane can be made.

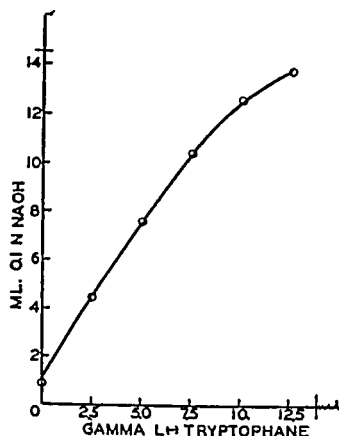


Fig. 1. Tryptophane standard curve (*Lactobacillus arabinosus*)

The chemical method used for the determination of tryptophane was based essentially on the method of Folin and Marenzi (8) with phosphomolybdotungstic acid for color development, which was measured in the Evelyn colorimeter with a 420 m $\mu$  filter. The chemical and comparative microbiological analyses of *l*(-)- or *dl*-tryptophane, which was treated with 5 N NaOH or Ba(OH)<sub>2</sub> respectively, are shown in Table II. These results indicate that 5 N Ba(OH)<sub>2</sub> but not 5 N NaOH treatment results in a marked destruction of tryptophane. Treatment with 14 per cent Ba(OH)<sub>2</sub>·8H<sub>2</sub>O caused little destruction of tryptophane.

To study the relationship between autoclaving time and the effect of 5 N Ba(OH)<sub>2</sub> and 5 N NaOH on tryptophane, samples of *l*(-)- and *dl*-tryptophane were treated with the respective reagents for varying lengths of time and analyzed for tryptophane by the chemical method. The results are graphically shown in Fig. 2 and demonstrate a progressive de-

struction of tryptophane by 5 N  $\text{Ba}(\text{OH})_2$ . That some tryptophane is adsorbed on the  $\text{BaSO}_4$  precipitate was evident, since about 2 per cent of

TABLE II  
Comparison of Microbiological and Chemical Assay of Tryptophane Treated by 5 N  $\text{NaOH}$  and 5 N  $\text{Ba}(\text{OH})_2$ , Respectively

Material treated, 10 mg.*	Method of treatment, at 121°	Tryptophane recovery	
		Microbiological assay	Chemical assay
		per cent	per cent
<i>l</i> (-)-Tryptophane	20 cc. 5 N $\text{NaOH}$ , 5 hrs.	90	95
<i>dl</i> -Tryptophane	20 " 5 " " 5 "	94	95
<i>l</i> (-)-Tryptophane	25 " 14% $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ , 7 hrs.	84	89
"	20 cc. 5 N $\text{Ba}(\text{OH})_2$ , 7 hrs.	53	52
<i>dl</i> -Tryptophane	20 " 5 " " 7 "	56	54
<i>l</i> (-)-Tryptophane	$\text{H}_2\text{O}$ , 5 hrs.	96	100
<i>dl</i> -Tryptophane	" 5 "	97	100

\* *dl*-Tryptophane activity for *Lactobacillus arabinosus* is only 50 per cent of *l*(-)-tryptophane, and in calculating the above recoveries only the *l* component was considered. For the chemical assay the *dl*- and *l*(-)-tryptophane have equal activity.

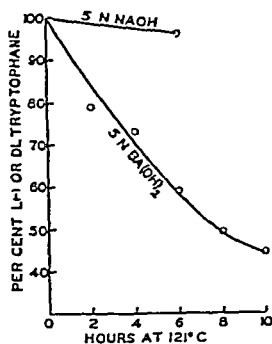


FIG. 2. Effect of time and 5 N alkali at 121° on tryptophane, as measured chemically.

the total tryptophane (by analysis) could be removed from the precipitate by further extraction of the precipitate with hot  $\text{H}_2\text{O}$ .

The results with 5 N  $\text{Ba}(\text{OH})_2$  and pure *l*(-)- or *dl*-tryptophane indicated that the microbiological assay results which had been obtained by

multiplying the actual assay value by 2 might also be dependent on a 50 per cent destruction of *l*(-)-tryptophane rather than its complete racemization. To test this idea samples of casein were autoclaved 5 hours at 121° with 5 *N* NaOH and 7 hours at 121° with 5 *N* Ba(OH)<sub>2</sub> or 14 per cent Ba(OH)<sub>2</sub>·8H<sub>2</sub>O, respectively. The samples were then analyzed for tryptophane by the chemical method (8) and it was found that the casein samples treated with 14 per cent Ba(OH)<sub>2</sub>·8H<sub>2</sub>O and 5 *N* Ba(OH)<sub>2</sub> contained 82 and 59 per cent as much tryptophane respectively as did the sample which had been autoclaved with 5 *N* NaOH.

These results substantiate the previous observations (9) that racemization of pure amino acids with alkali is induced with difficulty. Since racemization of *l*(-)-tryptophane is evident, however, when casein is hydrolyzed with 5 *N* NaOH (chemical values are twice as high as microbiological values), it appears that racemization precedes the hydrolytic cleavage of the peptide bond.

This difference in behavior of tryptophane which depends on whether it is free or combined in the protein molecule obviously makes for some difficulty in ascertaining recovery. Recovery of pure *l*(-)-tryptophane in the presence of casein (see Table I) was obtained, therefore, by assuming no racemization and subtracting the actual assay value for casein from the actual assay value of the recovery sample; i.e., casein plus *l*(-)-tryptophane. This study in general confirms the observation of Stokes *et al.* (6) that hydrolysis with 5 *N* NaOH provides an adequate and expedient method for liberating and racemizing tryptophane from proteins, although little advantage could be observed in extending the hydrolysis time beyond 5 hours.

Although the tryptophane values ( $\times 2$  to correct for racemization) obtained by 5 hours autoclaving at 121° with 5 *N* NaOH may not be absolute, they are consistent and representative, and consequently this method was used for liberating the tryptophane (when necessary) in the tryptophane balance experiments which follow. The indiscriminate application of this method of sample preparation is not warranted, however, inasmuch as some destruction of tryptophane is indicated and this destruction may increase, depending upon the sample that is analyzed.

*Tryptophane Balance Study*—Male rats of the Sprague-Dawley strain were used in the following experiments. The diets employed have been described in detail (1) and consisted of Diet A, which contained 2 parts of corn grits and 3 parts of the niacin-low synthetic ration, and Diet B, which was identical with Diet A, except for the addition of 1.5 mg. of niacin per 100 gm. of ration. Both diets contained 118 mg. per cent of tryptophane. Four rats, two on Diet A and two on Diet B, were placed in metabolism cages for a period of 4 weeks and careful records were kept of

weekly food consumption and gain in body weight. The urine and feces of each rat were collected separately and assayed for tryptophane. To determine the tryptophane content per gm. of rat, eight comparable rats were maintained on Diets A and B respectively, two rats on each diet were sacrificed at the end of each week of the metabolism study, and the entire carcass was assayed for tryptophane. This assured a fair comparison as to age and weight for calculating the tryptophane represented in the gm. gained per rat on the metabolism experiment.

Urine tryptophane was determined directly after the urine had been filtered at pH 4.4, readjusted to pH 7, and diluted to appropriate volume for tryptophane analysis. No effort was made to eliminate the possible interference of indole, since this or any other compound containing or derived from the indole nucleus which might be active for *Lactobacillus arabinosus* must presumably have originated from tryptophane. The niacin content of the urine was not determined since the amount of niacin excreted is too small to modify significantly the results in view of the relatively large amounts of niacin utilized for animal growth.

Feces were autoclaved for 1 hour with about 50 volumes of 1 N NaOH, after which an aliquot was taken for niacin determination (7). Another aliquot was further autoclaved with 5 N NaOH and assayed for tryptophane, as previously described.

The tryptophane content per gm. of rat was determined by autoclaving the entire carcass with 10 volumes of 1 N NaOH for 1.5 hours at 121°. This results in complete disintegration of the rat, except for bone fragments. An aliquot was then taken for niacin analysis (7) and another aliquot equivalent to 5 gm. of rat was made to 5 N by adding solid NaOH, autoclaved, and assayed for tryptophane as above. A remarkable consistency was noted in the tryptophane content of rat carcass regardless of diet, age, or size. The average tryptophane value for forty analyses was 1.70 mg. of tryptophane per gm. of rat with extreme values of 1.60 and 1.88 mg. per gm. The per cent of tryptophane utilization was determined by the following equation:

$$\frac{\text{Tryptophane per gm. rat} \times \text{gm. gained} + \text{tryptophane in urine and feces} \times 100}{\text{Tryptophane ingested (diet)}}$$

The 4 week data are summarized in Table III, Experiment I. In this experiment a comparison of Diets A and B was made over a 4 weeks growth period. In the second metabolism experiment, twelve rats were kept on Diet A (the deficient diet) for 4 weeks after weaning, at which time four were placed in metabolism cages for 1 week. Two of the four rats were then given Diet B (which contains niacin) for 1 week, while two rats were continued on Diet A. The tissue tryptophane on the compar-

able extra rats and the tryptophane in the feces and urine were determined as in Experiment I. It will be noted in Table III, Experiment II, that after 5 weeks on the deficient Diet A the rats made unusually large gains in

TABLE III  
*Effect of Niacin on Tryptophane Utilization*

Experiment No.	Diet	Rat No.	Gain per wk.*	Food intake	Tryptophane in urine	Weight of feces	Tryptophane in feces	Tryptophane in rat†	Tryptophane utilization	Food consumption per gm. gain
			gm.	gm. per wk.	γ per wk.	gm. per wk.	mg. per wk.	mg. per gm.	per cent	gm.
I	A	1	3.5	25.5	247	0.589	2.16	1.76	28.6	7.25
		2	8.5	46.0	326	0.744	2.36	1.70	31.6	5.41
		Average.....	6.0	35.7	287	0.666	2.26	1.73	30.1	6.33
	B	3	26.8	64.3	677	1.480	5.77	1.77	71.8	2.40
		4	27.2	66.5	724	1.105	5.03	1.74	68.2	2.44
		Average.....	27.0	65.4	700	1.292	5.40	1.76	70.0	2.42
II	A‡	5	5	29	368	0.530	1.62	1.67	30.6	5.8
		6	6	33	311	0.645	2.48	1.80	34.4	5.5
		Average.....	5.5	31	339	0.588	2.05	1.73	32.5	5.65
	A§	5	7	35	381	0.472	1.27	1.60	31.2	5.0
		6	10	40	144	0.865	2.64	1.70	41.8	4.0
		Average.....	8.5	37.5	262	0.668	1.95	1.65	36.5	4.5
	A‡	7	8	33	302	0.729	2.16	1.61	40	4.12
	B§	7	50	91	390	2.078	8.00	1.76	91	1.82
	A‡	8	5	30	563	0.540	1.64	1.60	28.6	6.00
	B§	8	43	76	510	1.697	6.90	1.84	93.8	1.77

\* The figures for Experiment I give the average gain per week for 4 weeks. The figures in Experiment II give the weekly gains for the 5th and 6th weeks after 4 weeks on Diet A.

† Analyses made of comparable rats on comparable diets.

‡ 5th week.

§ 6th week.

weight with a correspondingly high percentage of tryptophane utilization when they were given niacin. In addition to the fact that a high percentage of tryptophane can be accounted for in rats on Diet B, considerably less of this diet was consumed per gm. of gain in body weight than on Diet A.



Balance values similar to those obtained for tryptophane were calculated for niacin (Table IV). Even when the niacin content of the urine is disregarded, it is evident that over 100 per cent of the niacin ingested can be accounted for regardless of diet, age of rat, or stage of deficiency. This is in marked contrast to the tryptophane balance in which case about 30 and 70 per cent of the ingested tryptophane could be accounted for on the deficient and adequate diets respectively.

From the data in Tables III and IV it is evident that the tissue content of niacin and tryptophane is quite constant (*i.e.*, 33  $\gamma$  and 1.70 mg. per gm. respectively). In addition no significant difference can be noted between the niacin or tryptophane content per gm. of feces.

TABLE IV  
*Niacin Utilization*

Diet	Food intake	Niacin intake	Gain per wk.*	Niacin in rat	Weight of feces	Niacin in feces	Excess niacin†
	gm. per wk.	$\gamma$ per wk.	gm.	$\gamma$ per gm.	gm. per wk.	$\gamma$ per wk.	$\gamma$ per wk.
A, † Rat 1	25.5	68.8	3.5	32.5	0.589	85.7	122
" " 2	46.0	124	8.5	32.2	0.744	87.8	218
Average.....	35.7	96.4	6.0	32.4	0.666	86.8	170
B, ‡ Rat 3	64.3	1066	26.8	34.8	1.460	193	62
" " 4	66.5	1070	27.2	32.7	1.105	218	95
Average.....	65.4	1068	27.0	33.8	1.292	206	78

\* Average gain per week for 4 weeks.

† (Niacin per gm. of rat  $\times$  gm. gained) + (feces niacin) - (niacin ingested).

‡ Niacin content, 2.7  $\gamma$  per gm. of ration.

§ Niacin content 17.7  $\gamma$  per gm. of ration.

To examine further the relationship between niacin intake and output this ratio was calculated for the niacin-low synthetic basal, for the corn grits basal with dextrin as the carbohydrate in place of sucrose, and for the corn grits basal plus 50 mg. per cent of *l*(-)-tryptophane (1). The data obtained from the present metabolism study were used as a basis for the calculations which are compiled in Table V. (These calculations, while approximate, are representative and comparable.)

#### DISCUSSION

It is evident from the data (Table V) that the good growth and concomitant increase in total tissue niacin obtained on the niacin-low synthetic basal ration could be accounted for only by a mechanism of niacin syn-

thesis. Since the physiological processes of growth apparently involve a constancy of tissue components, growth will not occur in absence of any one essential component.

Although corn supplies some dietary niacin, this source would be wholly inadequate in view of the relatively large demands for tissue growth unless a supplementary synthetic mechanism were available. That this supplementary mechanism is impaired on such a diet is evident. When dietary niacin is added in adequate amounts to the corn ration, growth is resumed but apparently limited to the extent of dietary intake, since there is an almost exact balance between niacin intake and "output," with little niacin synthesis in evidence. In this case almost as much dietary niacin

TABLE V  
*Calculated Niacin Balance on Various Diets*

Diet used	Gain per wk.	Niacin balance*	
		Niacin intake	Niacin in tissues + feces
	gm.	γ per wk.	γ per wk.
Synthetic (sucrose)†.....	27	<10	1100
" + corn grits‡.....	6	100	280
" + " " + niacin.....	27	1068	1097
" + " " + tryptophane.....	27	194	1100
" (dextrin) + corn grits.....	21	194	943

\* Output (tissue niacin × gm. gained + feces niacin) - (niacin intake).

† Contains <0.1 mg. per cent of niacin. The composition of the ration has been described (1).

‡ Corn grits replaces 40 parts of entire ration (1).

was added as had formerly been synthesized on the niacin-low synthetic ration with approximately the same growth ensuing in both cases.

That the tryptophane content of the corn ration is adequate in the presence of added dietary niacin might be explained by the positive effect of niacin on the utilization of tryptophane. Good growth is obtained with non-corn niacin-low diets containing only 108 mg. per cent of tryptophane (3) on which niacin synthesis is evident, but fails when corn rations are used, although their tryptophane content is slightly higher (*i.e.*, 118 mg. per cent).

The good growth effected by the addition of tryptophane to corn rations could result from the action of tryptophane in improving niacin synthesis, which could then play a dual rôle of providing adequate niacin for tissue growth and improving tryptophane utilization. Whether tryptophane acts to stimulate niacin synthesis in the rat or whether it is an actual precursor of niacin remains to be determined.

It should also be noted that the above explanation would be applicable to the analogous syndrome which can be produced with non-corn rations.

### SUMMARY

Procedures for the liberation of tryptophane by the alkaline hydrolysis of proteins have been studied. Hydrolysis with 5 N NaOH at 121° for 5 hours provides an adequate and expedient method for liberating tryptophane with a minimum amount of destruction.

Racemization of pure *l*(-)-tryptophane does not take place when treated with 5 N NaOH or 14 per cent Ba(OH)<sub>2</sub>·8H<sub>2</sub>O. Similar treatment with 5 N Ba(OH)<sub>2</sub> appears to destroy both *l*(-)- or *dl*-tryptophane.

The utilization of tryptophane and niacin by rats on rations containing corn grits and corn grits plus niacin was determined by means of a balance study, and the results indicate that niacin improves the utilization of tryptophane from about 30 to about 70 per cent. Over 100 per cent of the ingested niacin was accounted for in all cases. The carcass content of niacin and tryptophane was remarkably constant.

An explanation is offered for the effectiveness of either niacin or tryptophane in counteracting the retarded growth of rats on corn rations.

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# A SPECTROPHOTOMETRIC METHOD FOR THE MEASUREMENT OF RIBONUCLEASE ACTIVITY

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Digestion of yeast nucleic acid by ribonuclease is accompanied by a shift in the ultraviolet absorption spectrum of the substrate towards the shorter wave-lengths (Fig. 1).

The shift is not due to the liberation of free acid which takes place during digestion (1, 2), since the effect of increasing the hydrogen ion concentration is to shift the absorption spectrum of yeast nucleic acid in a direction opposite to that caused by ribonuclease; namely, towards the longer wave-lengths (Figs. 2 and 3).

The shift in the ultraviolet spectrum caused by ribonuclease is most distinct in the region of 290 to 305  $m\mu$ , where the *gradual* decrease in the extinction (density)  $E = \log I_0/I$  during the initial stages of the digestion process can be readily measured. The determination of the rate of change in the absorption of the ultraviolet light can thus serve as a convenient method for measuring the concentration (activity) of the ribonuclease in the digestion mixture.

The effect of varying the concentration of ribonuclease on the value of  $E$  at 300  $m\mu$  is shown graphically in Fig. 4, A.

The value of  $E$  decreases linearly with time, at least during the initial phase of the reaction, the rate of decrease being nearly proportional to the concentration of enzyme in solution, as shown by the values for the slopes of the lines. Hence

$$-\frac{dE}{dt} = KC \quad (1)$$

is an approximate expression for the relationship between the change in  $E$  with time and the concentration  $C$  of the ribonuclease. However, the following relationship was found to hold for a wider range of concentration of ribonuclease; namely,

$$-\frac{dE}{dt} = KC(E - E_f) \quad (2)$$

where  $E$  = extinction (density) at any time,  $t$ , and  $E_f$  = final extinction. This equation is of the type corresponding to a unimolecular reaction, since the concentration of enzyme  $C$  is apparently constant during the reaction.

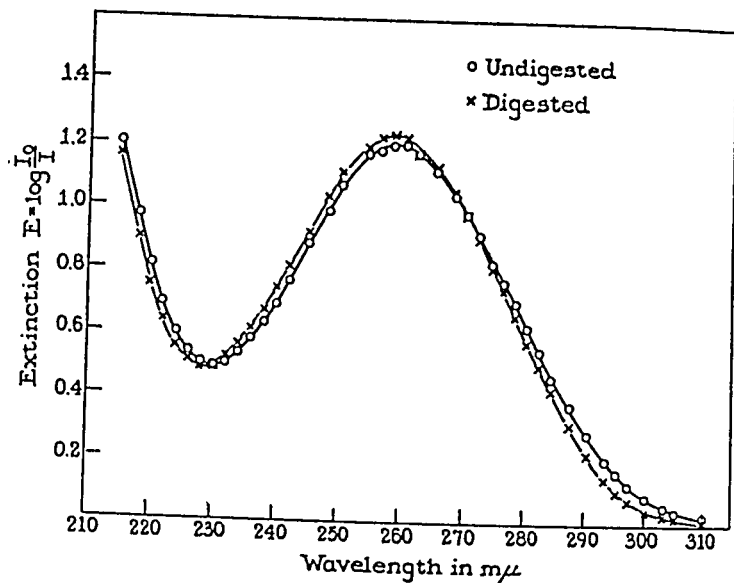


FIG. 1. The effect of ribonuclease on the ultraviolet absorption spectrum of yeast nucleic acid at pH 5.0. Concentration of nucleic acid (Boehringer's yeast nucleic acid, purified by reprecipitation with glacial acetic acid (1), was used in most of the tests) 0.05 mg. per ml. of 0.05 M acetate buffer, pH 5.0. Concentration of crystalline ribonuclease (1) 0.00025 mg. per ml. The length of absorbing solution = 1 cm. The plotted values of  $E$  are observed readings of density  $\log I_0/I$  of the solution.

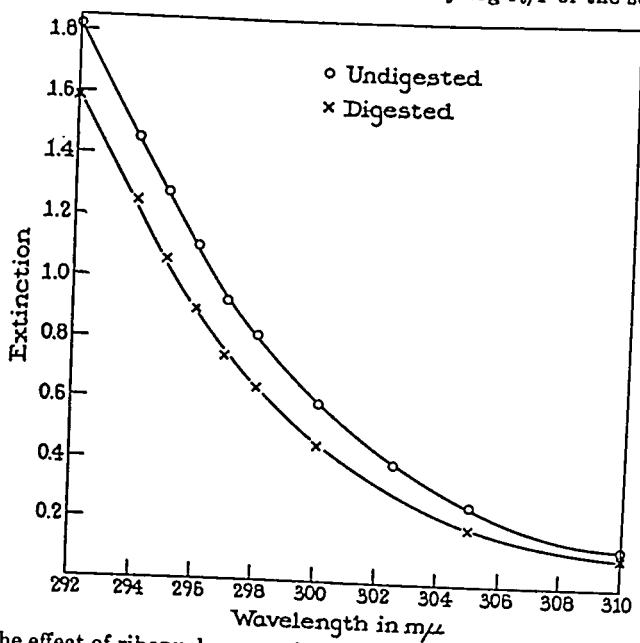


FIG. 2. The effect of ribonuclease on the extinction (density) of a solution of yeast nucleic acid in the range of 292 to 310 mμ at pH 5.0. Concentration of nucleic acid 0.5 mg. per ml. Concentration of ribonuclease 0.0025 mg. per ml.

Equation 2, when integrated, gives

$$-\log (E - E_f) = \frac{KC}{2.3} t - \log (E_0 - E_f) \quad (2, a)$$

When  $\log (E - E_f)$  is plotted against  $t$ , a straight line is obtained, the slope of which equals  $-KC/2.3$ , while  $\log (E_0 - E_f)$  is the intercept at  $t = 0$ .

The value of  $E_f$  is generally reached within 1 to 3 hours of digestion and is independent of the concentration of ribonuclease used.  $K$ , the proportionality constant, is a measure of the units of ribonuclease activity per unit

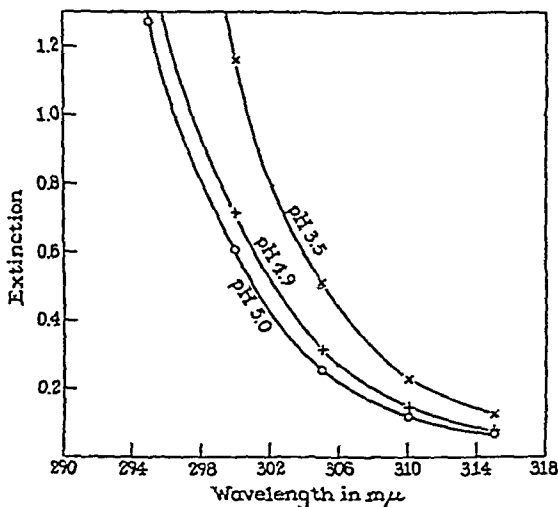


FIG. 3. The effect of pH on the extinction (density) of a solution of yeast nucleic acid in the range of 294 to 315  $m\mu$ .

concentration of enzyme and is obtained by plotting  $\log (E - E_f)$  versus  $t$  (Fig. 4, B), multiplying the slope of the straight line drawn through the plotted points by  $-2.3$ , and dividing by the concentration of the ribonuclease in solution.

The unit of ribonuclease activity is defined here as the amount of enzyme which is capable of causing a decrease of 100 per cent per minute in the value of  $E_0 - E_f$  of the substrate at  $25^\circ$ . The substrate consists of 0.05 per cent solution of yeast nucleic acid in 0.05 M acetate buffer, pH 5.0.  $E_0 - E_f$  is the maximum possible change in the extinction at 300  $m\mu$ .

#### EXPERIMENTAL

The method of measuring ribonuclease activity by the spectrophoto-

metric method consists simply in mixing rapidly 2 ml. of 0.1 per cent solution of yeast nucleic acid in 0.1 M acetate buffer, pH 5.0, with 2 ml. of a dilute solution of ribonuclease in water, both at about 25°. The time of

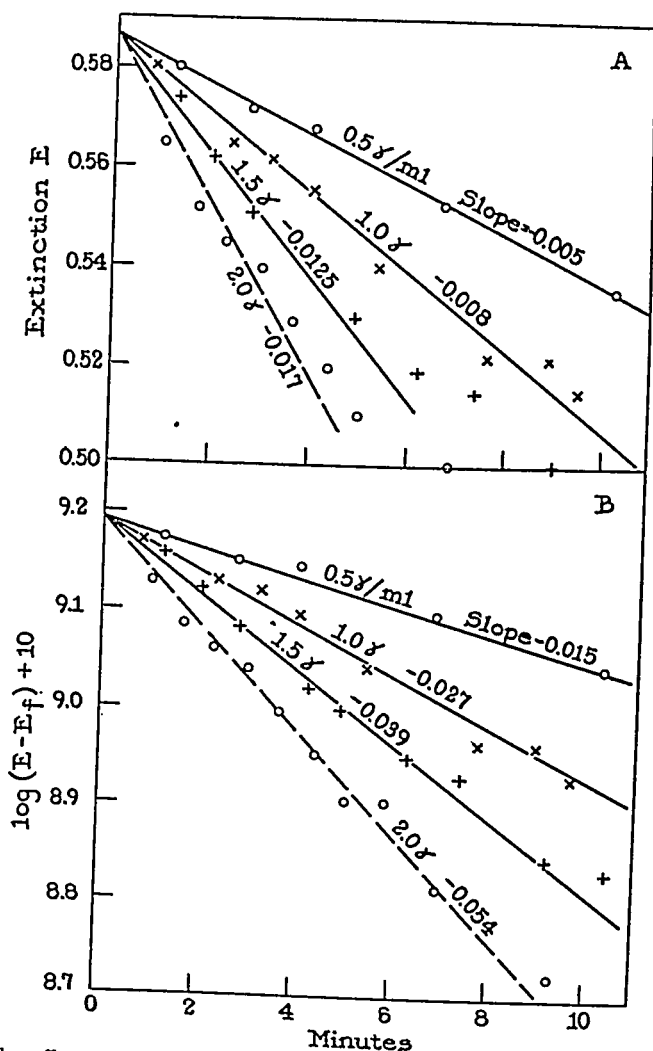


FIG. 4. The effect of concentration of ribonuclease on the extinction (density) of 0.05 per cent solution of yeast nucleic acid, pH 5.0, at 300  $m\mu$ .

mixing is carefully observed, or still better, a stop-watch is started at the moment of mixing. The mixing is more prompt if substrate and enzyme are prepared in separate tubes and then mixed by pouring rapidly from one tube to the other, and back and forth.

The mixed solution is immediately transferred into a quartz cell of 1 sq.



cm. inner cross-section, and extinction (density) readings at 300  $m\mu$  are taken every minute or longer for about 10 minutes, depending on the rate of drop in extinction.

TABLE I

*Effect of Ribonuclease on Ultraviolet Light Absorption at 300  $m\mu$  of Various Commercial Preparations of Yeast Nucleic Acid*

Digestion mixture, 2 ml. of 0.1 per cent nucleic acid in 0.1  $M$  acetate buffer solution, pH 5.0, plus 2 ml. of solution of 0.005 mg. of crystalline ribonuclease per ml. of  $H_2O$ . The values of  $E$  are observed densities ( $\log I_0/I$ ).

Yeast nucleic acid	$E_i$	$E_f$	Slope of $\log (E_i - E_f)$ versus $t$	Activity, ribonuclease units per ml.	Ribonuclease units per mg. ribonuclease
Boehringer.....	0.564	0.406	-0.047	0.108	43
Standard Brands.....	0.563	0.423	-0.044	0.101	40
Merck.....	0.516	0.412	-0.040	0.092	37
Na nucleinate, Merck.....	0.536	0.356	-0.040	0.092	37
" " Schwarz Laboratories.....	0.578	0.362	-0.038	0.087	35

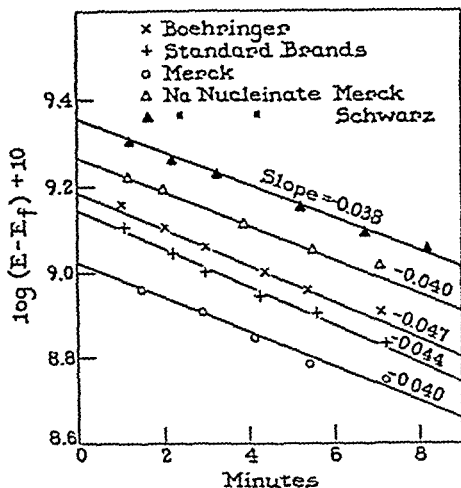


FIG. 5. Various preparations of yeast nucleic acid

A control solution consisting of a mixture of 2 ml. of the yeast nucleic acid and 2 ml. of water is used as a check on the instrument. The control solution, if not contaminated with a trace of enzyme, generally does not show any drop in extinction, even after standing for several hours at room temperature. All the measurements are taken against 0.05  $M$  acetate

buffer, pH 5.0, as a standard for  $I_0$ . The readings are plotted either directly against  $t$  in minutes or as  $\log (E - E_f)$  versus  $t$ , depending on the rapidity of the reaction.

The ribonuclease activity per ml. of solution is equal in the first case to the slope of the line drawn through the plotted points of  $E$  versus  $t$ , divided by the value of  $E_0 - E_f$ , while in the second case it equals the slope of the line drawn through the plotted points of  $\log (E - E_f)$  versus  $t$ , multiplied by  $-2.3$ . Dividing by the factor  $E_0 - E_f$  in the first case is necessary in order to express the activity in units comparable to those of the logarithmic expression, as defined in Equation 2, *a*.

*Effect of Using Various Preparations of Yeast Nucleic Acid*—Several commercial samples of yeast nucleic acid were tried simultaneously. The results are given in Fig. 5 and summarized in Table I. The difference between the various preparations is not very significant. It is convenient, however, to use preparations in which the difference between  $E_0$  and  $E_f$  is greatest. A Beckman quartz spectrophotometer has been used for the extinction measurements.

#### SUMMARY

A convenient method for the quantitative determination of ribonuclease activity is described. The method is based on the fact that ribonuclease causes a gradual shift in the ultraviolet absorption spectrum of yeast nucleic acid towards the shorter wave-lengths.

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# STEROIDS DERIVED FROM BILE ACIDS

## IV. 3,9-EPOXY- $\Delta^9,11$ -CHOLENIC ACID AND CLOSELY RELATED COMPOUNDS

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It has been shown that the  $C_{12}$ -hydroxyl group in 3( $\alpha$ ),12-dihydroxy- $\Delta^9,11$ -choleonic acid is easily replaced with an acetoxyl or methoxyl group (1). Further investigation has revealed the fact that through the action of hydrogen chloride the  $C_{12}$ -hydroxyl, methoxyl, and acetoxyl compounds are readily converted into the same allyl chloride.<sup>1</sup> This derivative, even under mild conditions, loses halogen acid to give a new product which appears to contain an oxygen bridge between  $C_2$  and  $C_3$ . The preparation and properties of this new product and related compounds are described in this paper (Figs. 1 and 4).

A chloroform solution of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9,11$ -cholelate (II)<sup>2</sup>(1) was saturated at 0° with dry hydrogen chloride and after removal of the solvent a product was separated by crystallization from petroleum ether which possessed the properties of the expected allyl halide, methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,11$ -cholelate (IV). The chlorine in this compound is assigned to  $C_{12}$ , since treatment with a concentrated solution of sodium hydroxide gave 3( $\alpha$ ),12-dihydroxy- $\Delta^9,11$ -choleonic acid (I) (1, 2). When dissolved in methanol, IV underwent a replacement of chlorine by methoxyl to give the known  $C_{12}$ -methoxyl derivative (II). Methyl 3( $\alpha$ )-acetoxyl-12-methoxy- $\Delta^9,11$ -cholelate (VIII) and methyl 3-keto-12-methoxy- $\Delta^9,11$ -cholelate (XXV) have also been converted into derivatives with halogen at  $C_{12}$  (VI and XXIV respectively) by the foregoing method, and treatment of these compounds in aqueous acetone with a silver salt yielded the  $C_{12}$ -hydroxy derivatives (VII and XXIII) (1).

When methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,11$ -cholelate (IV) in chloroform solution was not maintained in an anhydrous state, as previously described, but was washed with water and a dilute solution of sodium bicarbonate, the product obtained (XI) did not contain halogen.<sup>3</sup>

From analogy with the formation of 3( $\alpha$ ),12-dihydroxy- $\Delta^9,11$ -choleonic

<sup>1</sup> Hydrobromic acid forms the corresponding  $C_{12}$ -bromo compound.

<sup>2</sup> Roman numerals refer to the various compounds in Figs. 1 and 4.

<sup>3</sup> The same compound (XI) was subsequently formed by treatment of the chloroform solution of IV with pyridine.



the chloroform was not III. Analysis of the product indicated the presence of only 3 atoms of oxygen and determination of the molecular weight showed that the molecule was a monomer. Absence of selective absorption in the ultraviolet region excluded the possibility that loss of hydrogen halide had resulted in the formation of a conjugated diene, but the presence of an active double bond was shown by the utilization of 1 mole of perbenzoic acid to give an oxide (XIV), and by the absorption of 1 mole of bromine to give a mixture from which two dibromo derivatives, XII and XIII, were isolated.

The absence of a hydroxyl group at  $C_3$  was shown by failure of the acid X to form an acetyl derivative with acetic anhydride and pyridine and by recovery of the dibromide XII unchanged from a solution of excess chromic acid in acetic acid.

The new product (X) was also recovered unchanged from a strong solution of sodium hydroxide but studies of the behavior of the ester XI in acid mediums indicated that an allylic system was present which possessed properties similar to those of the allylic compound methyl 3( $\alpha$ ),12-dihy-

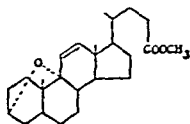


Fig. 2

droxy- $\Delta^9,^{11}$ -cholenate (III) and its  $C_{12}$ -methoxyl derivative (II). In chloroform both II and XI were converted with hydrogen chloride into methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV). In acetic acid and a trace of sulfuric acid II and XI yielded methyl 3( $\alpha$ )-hydroxy-12-acetoxy- $\Delta^9,^{11}$ -cholenate (XVI). On oxidation with chromic acid in acetic acid, both II and X yielded small amounts of the known methyl 3,12-diketo- $\Delta^9,^{11}$ -cholenate (XXI) (1) and in methanol and a trace of mineral acid XI was converted into II.

On the basis of the evidence which has been presented we suggest the structure shown in Fig. 2 as the most probable for product XI.

Several arguments may be advanced in support of this formulation. First, a simple mechanism exists for the formation of XI from the allyl halides IV and V by loss of halogen acid. Removal of the halogen at  $C_{12}$  through solvation would be expected to give rise to a carbonium ion with the residual charge distributed between  $C_3$  and  $C_{12}$ . The resultant hybrid can yield a stable structure by formation of the six-membered cyclic ether with loss of a proton from the hydroxyl group at  $C_3$  (Fig. 3).<sup>4</sup>

<sup>4</sup> The isolation of 3( $\alpha$ ),12-dihydroxy- $\Delta^9,^{11}$ -cholenic acid (I) from treatment of methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV) with concentrated alkali, which

Evidence in favor of the reaction mechanism suggested for the formation of XI is furnished by derivatives of methyl 3( $\alpha$ )-hydroxy-12-halo- $\Delta^{9,11}$ -cholenate (IV and V) which cannot form the 3,9-epoxy structure but which can yield carbonium ions of the type under discussion by loss of halogen. Two such compounds are methyl 3( $\alpha$ )-acetoxy-12-chloro- $\Delta^{9,11}$ -cholenate (VI) and methyl 3-keto-12-bromo- $\Delta^{9,11}$ -cholenate (XXIV). The carbonium ions which result from removal of the halogen atoms from these compounds are stabilized by loss of a proton and formation of conjugated dienes. The results of this investigation will be reported in a subsequent paper.

Further evidence for the 3,9-epoxy structure is given by construction of Stuart models (4). Experimental evidence for the configuration of the bile acids (5-8), although not conclusive, indicates that the fusion between Rings A and B is *cis* as in *cis*-decalin, and that the hydroxyl group at C<sub>3</sub> is  $\alpha$ ; that is, that it lies on the side of the molecule opposite to the methyl group at C<sub>10</sub> (9). The model shows that carbon atoms 3 and 9 are in a

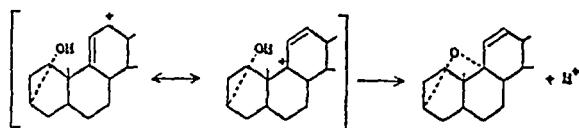


FIG. 3

relation such that closure of a cyclic ether of the type discussed can be effected without distortion. A model with a cyclic ether linkage between C<sub>3</sub> and C<sub>12</sub> cannot be made and it is also impossible to form a model with a 3,9-epoxy structure if the Rings A and B are *trans*, or with the cyclic ether on the same side of the Ring A as the methyl group at C<sub>10</sub>. These results furnish good confirmation of the previous assumptions in regard to the configuration of the bile acids.

The most convincing evidence for the structure of XI was obtained by the preparation of the 11,12-oxide (XXVII). This yielded two of the four possible glycols at 11,12 (XXIX and XXXI) and both of these glycols in turn gave the same  $\alpha$ -diketone (XXXII) on careful oxidation with chromic acid (Fig. 4).

Absorption in the ultraviolet region by the diketone showed a striking similarity to that reported by Barnett and Reichstein (10) for the keto form of 11,12-diketocholelic acid. The shift to the longer wave-length with maxima at 295 and 376  $m\mu$  and  $\log \epsilon$  2.018 and 1.590, respectively, exhib-

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has been previously mentioned, may be interpreted as a direct replacement of the halogen with the hydroxyl group (3).

ited by our compound may be attributed to the influence of the adjacent atom of oxygen at C<sub>9</sub> (Fig. 5).

It has been suggested that the formation of both the 3,9-epoxy structure and the diene from allyl halides at C<sub>12</sub> is through removal of halogen and formation of a carbonium ion. Stabilization of the ion is accomplished in each instance through loss of a proton but the conditions under which halogen acid is lost to form the epoxide in one case, or the diene in the other,

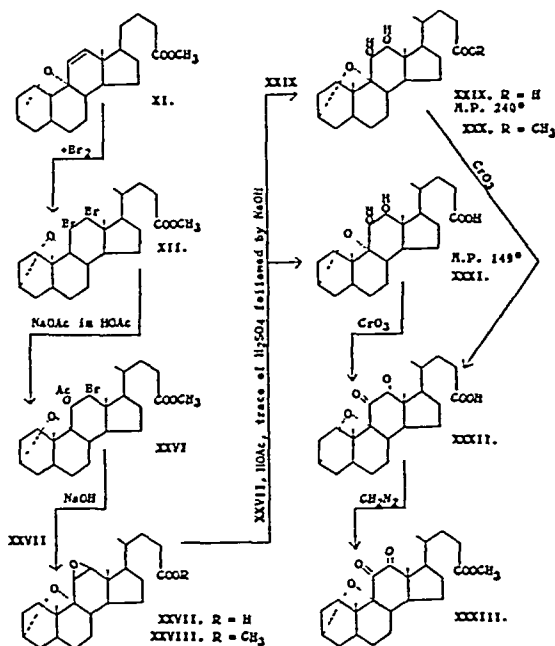


FIG. 4

are quite different. The 3,9-cyclic ether is formed at room temperature within a matter of minutes when a chloroform solution of methyl 3(α)-hydroxy-12-bromo-Δ<sup>9,11</sup>-cholenate (V) is washed with water. In contrast, methyl 3-keto-12-bromo-Δ<sup>9,11</sup>-cholenate (XXIV) and methyl 3(α)-acetoxy-12-chloro-Δ<sup>9,11</sup>-cholenate are entirely stable in a mixture of chloroform and water; the dienes are formed at the boiling point of pyridine.

Another example of the difference in lability of the halogen at C<sub>12</sub> is given by a comparison of the behavior of the closely related compounds methyl 3(α)-hydroxy-12-bromo-Δ<sup>9,11</sup>-cholenate (V) and the corresponding 3-keto derivative (XXIV) in methanol. The bromine in V was replaced with

the methoxyl group at  $0^\circ$  to give methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9, 11$ -cholenate (II), but XXIV was recovered unchanged after a methanolic solution of the compound had been heated to boiling. The bromine in XXIV was replaced with the methoxyl group after addition of silver nitrate to the methanolic solution at room temperature.

Reduction of the double bond in 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholenic acid (11, 12) in acetic acid with hydrogen and platinum to give lithocholic acid occurs

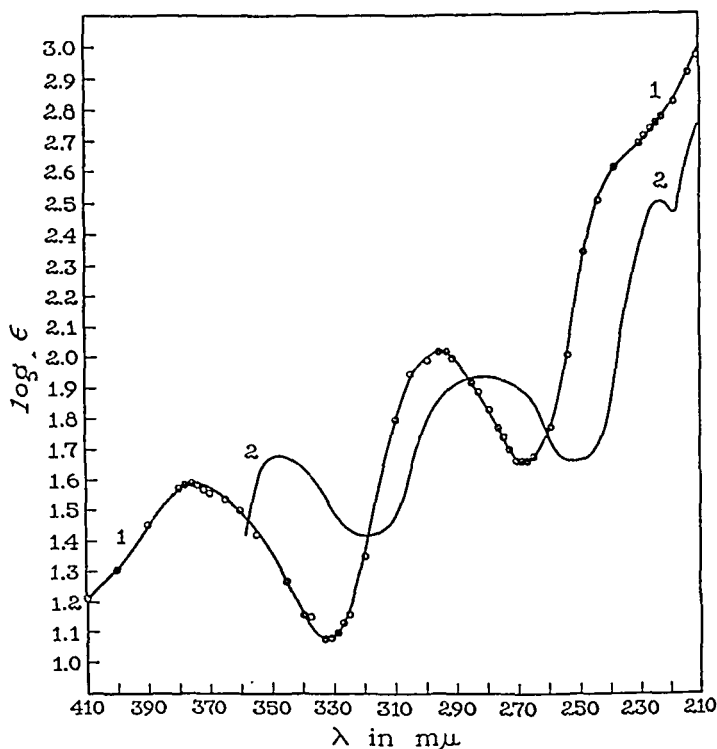


FIG. 5. Absorption spectra of (Curve 1) methyl 3,9-epoxy-11,12-diketocholanate and (Curve 2) methyl 11,12-diketocholanate, reproduced from the paper by Barnett and Reichstein (10).

rapidly and it was anticipated that the reduction of the double bond  $C_{11}:C_{12}$  in methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (XI) would give methyl 3,9-epoxycholanate. However, in neutral ethanol neither the double bond nor the epoxy structure of XI was altered by hydrogen and Adams' platinum catalyst. Addition of acetic acid to the solution produced rapid uptake of 1 mole of hydrogen, after which absorption of hydrogen almost ceased. After hydrolysis of the ester the product which was separated was not 3,9-epoxycholenic acid but was shown to be 3( $\alpha$ )-hydroxy- $\Delta^9, 11$ -cholenic acid (XVII) (13, 14). This is described in a later paragraph.



It was subsequently shown that the absorption of hydrogen occurred with formation of XVII when 3,9-epoxy- $\Delta^{11}$ -cholenic acid (X) in neutral ethanol was used in place of its ester, and that small amounts of a weak organic acid induced the reduction of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (XI) in neutral ethanol. For example 1 mm of the ester XI and 0.5 mm of lithocholic acid in neutral ethanol absorbed 98 per cent of 1 molar equivalent of hydrogen in 22.25 hours. When 1 mm of lithocholic acid was present but when, in addition to the lithocholic acid, a basic substance, ethylmorpholine, was added in an amount equal to the acid, less than 2 per cent of 1 molar equivalent of hydrogen was absorbed by the ester (XI) during an interval of 4 hours under identical conditions.

These observations suggest that the epoxide ring is opened to give methyl 3( $\alpha$ )-hydroxy-12-ethoxy- $\Delta^9,^{11}$ -cholenate or an equivalent compound, prior to hydrogenolysis. However, the absorption of hydrogen and cleavage of the cyclic ether in acetic acid proceed at a rate far greater than can be expected from observations on the opening of the 3,9-epoxide ring through the influence of acetic acid alone, and the absorption of hydrogen by XI is at a rate much greater than is the rate of the removal of the hydroxyl, methoxyl, or acetoxyl group at  $C_{12}$  through hydrogenolysis.

The acid XVII (13, 14) was identified by reduction to lithocholic acid (15) and by formation of an acetoxyl ester (XIX) and an oxide (XX). The methyl ester (XVIII) was found to be dimorphous, with melting points of 105.5° and 113°, and was thus differentiated from methyl lithocholate, which melts at 129–130° without previous softening or change in crystal structure (14, 16).

In discussion of this series of compounds the structure of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9,^{11}$ -cholenate (II) and the closely related derivatives with hydroxyl, chlorine, or acetoxyl at  $C_{12}$  (III, IV, and XVI, respectively) was left without proof for the position of the double bond. The position of the substituent (hydroxyl, acetoxyl, methoxyl, or halogen) could be at  $C_{12}$  with a double bond  $C_9:C_{11}$  as suggested, or at  $C_9$  with a double bond  $C_{11}:C_{12}$ . Although rigid proof of the position of the substituent is lacking, treatment of the compounds with bromine provides evidence for the position of the double bond.

In a chloroform solution the known methyl 3( $\alpha$ )-hydroxy- $\Delta^9,^{11}$ -cholenate and its  $C_{12}$ -keto derivative and the compounds II, III, IV, and XVI to which the double bond has been assigned the  $C_9:C_{11}$  position do not readily utilize small amounts of bromine. However, methyl 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholenate and its 3,9-epoxy derivative (XI) rapidly add 1 mole of bromine and form dibromo derivatives which are stable in a two-phase mixture of chloroform and aqueous sodium bicarbonate.

It was furthermore observed that, in the presence of a large excess of bromine, absorption of halogen by the compounds II, III, IV, and XVI is

rapid and apparently involves addition to the double bond  $C_9:C_{11}$ . The initial product is not stable; loss of hydrogen bromide and utilization of more than 1 mole of bromine occur even at  $-50^\circ$ , and at room temperature almost all the bromine taken up is removed as halogen ion in a two-phase system of chloroform and aqueous sodium bicarbonate. The instability of the bromo derivatives of the  $\Delta^9,^{11}$  series is in strong contrast to the properties of bromo derivatives from the  $\Delta^{11}$  compounds and furnishes evidence for the structure assigned with the double bond  $C_9:C_{11}$ .

The preparation of steroids with the 3,9-epoxy structure provides additional evidence for the configuration of the bile acids. Further study of compounds in this series promises to yield contributions of both theoretical and practical importance. The use of the dibromo derivative (XII) as an intermediate for the preparation of steroids which have an atom of oxygen at  $C_{11}$  will be reported in subsequent papers.

#### EXPERIMENTAL<sup>6</sup>

All melting points were taken on the Fisher-Johns apparatus.

*3( $\alpha$ ), 12-Dihydroxy- $\Delta^9,^{11}$ -cholenic Acid (I) (1, 2) from IV*—846 mg. of methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV) were slowly added to a solution composed of 15 cc. of methanol, 5 cc. of water, and 5 cc. of 18 N sodium hydroxide which was heated to  $60^\circ$  and stirred mechanically. The crystals of IV dissolved rapidly and after 15 minutes the methanol was removed under reduced pressure, the volume was made to about 75 cc. by addition of water, 20 cc. of benzene were added, and the solution was acidified with 20 cc. of 5 N sulfuric acid. 362 mg. of material which separated from the benzene were dissolved in a small volume of methanol and recrystallized by addition of benzene. The product melted at  $149-150^\circ$ , resolidified, and melted at  $192-193^\circ$ . Crystallized from acetone and placed on the block heated to  $193^\circ$  the compound melted at  $195-196^\circ$  and did not depress the melting point of 3( $\alpha$ ), 12-dihydroxy- $\Delta^9,^{11}$ -cholenic acid.  $[\alpha]_D^{25} = +104^\circ \pm 2^\circ$  (30.1 mg. in 3 cc. of methanol). The yield of I ( $C_{24}H_{38}O_4 \cdot \frac{1}{2}C_6H_6$ ) was 44 per cent of the theoretical amount.

*Methyl 3( $\alpha$ )-Hydroxy-12-methoxy- $\Delta^9,^{11}$ -cholenate (II) (1) from IV*—Methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV) is converted to II under the same conditions described for "II from VI."

*II from V*—100 mg. of methyl 3( $\alpha$ )-hydroxy-12-bromo- $\Delta^9,^{11}$ -cholenate (V) were added to 4.0 cc. of methanol at  $0^\circ$  and the solution was allowed to stand in an ice bath 21 hours. Chipped ice was added; the crystals which

\* Some of the compounds described in this paper were analyzed in the laboratory of Merck and Company, Inc., Rahway, New Jersey; the remainder was analyzed by Mr. William Saschek in the Department of Biochemistry of Columbia University, New York.

separated (83 mg.) melted at 156–157°. After recrystallization from methanol the product melted at 161–162° and did not depress the melting point of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9,^{11}$ -cholenate. The hydrogen bromide in the water was 98 per cent of the theoretical amount.

*II from V*—500 mg. of methyl 3( $\alpha$ )-hydroxy-12-bromo- $\Delta^9,^{11}$ -cholenate (V) which contained 0.5 mole of hydrogen bromide of crystallization were added to 20 cc. of methanol at 0°. 402 mg. (98 per cent of the theoretical amount) of material separated after addition of chipped ice. The melting point was 160–161° and there was no depression when the sample was melted after admixture with II. 98 per cent of the theoretical amount of hydrobromic acid was in the solution.

*II from VI*—A solution of 435 mg. of methyl 3( $\alpha$ )-acetoxy-12-chloro- $\Delta^9,^{11}$ -cholenate (VI) in 1.5 cc. of benzene was cooled to 5°, diluted with 5 cc. of methanol, and allowed to stand at 5° for 47 hours. Benzene was added, and the organic phase was washed with water, dried with sodium sulfate, and concentrated to dryness under reduced pressure. The residue crystallized from methanol to give 160 mg. of product which melted at 157–158°. An additional 158 mg. which melted at 158–159.5° were obtained by addition of water. After recrystallization from methanol the product melted at 160–162° and did not depress the melting point of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9,^{11}$ -cholenate.  $[\alpha]_D = +131^\circ \pm 2^\circ$  (31.0 mg. in 3.00 cc. of methanol).

*II from X*—3,9-Epoxy- $\Delta^{11}$ -cholenic acid is converted to II under the conditions described in the following paragraph, since the carboxyl group is rapidly esterified in methanol with a trace of mineral acid.

*II from XI*—773 mg. of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (XI) were dissolved in 10 cc. of methanol, the solution was cooled to about 15°, and 0.20 cc. of 5 N hydrochloric acid was added. After the solution had stood at room temperature 24 hours, it was seeded with II and diluted with a little water. The crystals which separated (712 mg.) melted at 159–160°. After recrystallization from methanol the product melted at 162–163° and did not depress the melting point of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9,^{11}$ -cholenate (II).

*Methyl 3( $\alpha$ ),12-Dihydroxy- $\Delta^9,^{11}$ -cholenate (III) (1, 2) from IV*—845 mg. of methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV), 50 cc. of acetone, 20 cc. of water, 290 mg. of silver carbonate, and 25 gm. of glass beads were placed in a glass-stoppered container and the mixture was rotated for 17 hours. The silver chloride which formed was 100 per cent of the theoretical amount. The filtrate was concentrated under reduced pressure to about 25 cc. and cooled in ice. The resulting crystals (788 mg.) melted at 82–84°, and after two recrystallizations from methanol-water, the product (431 mg.), when placed on the melting point apparatus

at 95°, melted at 97–98°.⁴ When the product was mixed with a sample of III and heated as described, the melting point was not depressed.  $[\alpha]_D = +99^\circ \pm 2^\circ$  (28.6 mg. in 3.00 cc. of methanol).

*III from V*—2.34 gm. of methyl 3(α)-hydroxy-12-bromo-Δ⁹,¹¹-cholenate (V) were treated as described under the preparation of III from IV, and the same molar ratio of materials was used. 1.62 gm. of crystals which melted at 82–85° were obtained. After recrystallization from ethyl acetate-ligroin and from methanol-water 792 mg. of product were obtained which, when placed on the melting point apparatus at 95°, melted at 97–98° and did not depress the melting point of III.  $[\alpha]_D = +97^\circ \pm 2^\circ$  (32.0 mg. in 3.00 cc. of methanol).

*Methyl 3(α)-Hydroxy-12-chloro-Δ⁹,¹¹-cholenate (IV) from II*—A solution of 14.00 gm. of methyl 3(α)-hydroxy-12-methoxy-Δ⁹,¹¹-cholenate (II) (1) in 100 cc. of dry, alcohol-free chloroform was cooled in an ice bath and saturated with dry hydrogen chloride over a period of 15 minutes. The solution was allowed to stand in the ice bath 30 minutes and was then concentrated under reduced pressure to about 25 cc. and diluted with 100 cc. of petroleum ether.⁷ The first crop of crystals (m.p. 120–129°) weighed 12.34 gm.; the second (m.p. 117–122°), 1.16 gm. The analytic sample, prepared by recrystallization from absolute ether-petroleum ether, melted at 119–124°.⁶  $[\alpha]_D = 149^\circ \pm 2^\circ$  (29.2 mg. in 3.00 cc. of chloroform).

$C_{25}H_{40}O_2Cl$	Calculated.	C 70.98,	H 9.29,	Cl 8.38
	Found.	" 71.05,	" 9.47,	" 8.28

*IV from XI*—1.40 gm. of methyl 3,9-epoxy-Δ¹¹-cholenate (XI) in 10 cc. of chloroform were treated in an ice bath with dry hydrogen chloride for 15 minutes. After 30 minutes the chloroform was concentrated under reduced pressure almost to dryness. Crystals (1.30 gm.) separated after addition of petroleum ether. The melting point was 122–124°. A second crop of 161 mg. melted at 120–126°.⁸ The product did not depress the melting point of methyl 3(α)-hydroxy-12-chloro-Δ⁹,¹¹-cholenate which was prepared from II.  $[\alpha]_D = +151^\circ \pm 2^\circ$  (30.8 mg. in 3.00 cc. of chloroform).

*IV from XVI*—447 mg. of methyl 3(α)-hydroxy-12-acetoxy-Δ⁹,¹¹-cholenate (XVI) (1) were dissolved in 10 cc. of chloroform, the solution was cooled to 0°, and 10 cc. of cold concentrated hydrochloric acid were added. The mixture was stirred mechanically for 1 hour at 0° and the chloroform phase was separated and washed with 10 cc. of concentrated hydrochloric

⁴ The methyl esters III, IV, V, and VII were difficult to prepare in a form which had a constant and sharp melting point. Recrystallization frequently lowered the melting point. The size of the crystals, the rate of heating, and the temperature when the sample was placed on the heating block were important factors.

⁷ Throughout this paper petroleum ether will designate a commercial product, b.p. 40–60°.

acid. The chloroform solution was dried over sodium sulfate, diluted with ligroin,<sup>8</sup> and concentrated under reduced pressure. Three crops of crystals separated: 148 mg. which melted at 120–132°, 150 mg. which melted at 120–138°, 18 mg. which melted at 119–123°. When the first two crops were combined and recrystallized from chloroform-ligroin, the product melted at 120–124° and did not depress the melting point of IV from II.  $[\alpha]_D = +148^\circ \pm 2^\circ$  (30.9 mg. in 3.00 cc. of chloroform).

*Methyl 3( $\alpha$ )-Hydroxy-12-bromo- $\Delta^9$ -<sup>11</sup>-cholenate (V) from II*—A solution of 12.56 gm. of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9$ -<sup>11</sup>-cholenate (II) (1) in 75 cc. of chloroform was chilled in an ice bath and a stream of dry hydrogen bromide was passed through the solution for 20 minutes. 100 cc. of ligroin were added. The solution was concentrated until it became turbid and was allowed to stand a few minutes. The first crop of crystals (m.p. 136.5–138°) weighed 5.35 gm. By further concentration of the solution successive crops of crystals were obtained: 0.94 gm., m.p. 135–137°; 2.98 gm., m.p. 131–133°; 1.52 gm., m.p. 137–137.5°; 2.94 gm., m.p. 135–136°. Treatment of V with methanolic alkali yielded more than the theoretical amount of bromide ion and further investigation disclosed the fact that the first crop of crystals contained hydrogen bromide in the proportion of 2 moles of V with 1 mole of halogen acid.  $[\alpha]_D = +205^\circ \pm 2^\circ$  (31.4 mg. of the first crop in 3.00 cc. of chloroform). The analytic sample was dried in a vacuum of 0.1 mm. for 6 hours at room temperature.

$C_{25}H_{33}O_2Br \cdot \frac{1}{2}HBr$ .	Calculated.	C 59.11, H 7.84, Br 23.60
	Found.	" 59.24, " 7.69, " 23.38
		" 59.21, " 7.74

Later crops of V sometimes separated without hydrogen bromide of crystallization. In one experiment the crystals melted at 137–138°; however, recrystallization invariably yielded material which melted at a lower point and over a wider range.  $C_{25}H_{33}O_2Br$ , calculated, Br 17.1, found 16.8.  $[\alpha]_D = +209^\circ \pm 2^\circ$  (29.9 mg. in 3 cc. of chloroform).

*V from III*—600 mg. of III in 5 cc. of chloroform treated with hydrogen bromide as described in the preceding paragraph gave crystals which melted at 135.5–136.5° when placed on the block at 125° and did not depress the melting point of V which did not contain hydrobromic acid of crystallization.  $C_{25}H_{33}O_2Br$ , calculated, Br 17.09, found 17.30.  $[\alpha]_D = +213^\circ \pm 2^\circ$  (31.3 mg. in 3 cc. of chloroform).

*V from XI*—Methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (XI) was converted to methyl 3( $\alpha$ )-hydroxy-12-bromo- $\Delta^9$ -<sup>11</sup>-cholenate (V) with hydrobromic acid under the conditions described for the preparation of the corresponding  $C_{12}$ -chloro compound (IV) by treatment of XI with hydrochloric acid.

<sup>8</sup> Throughout this paper ligroin will designate a commercial product, b.p. 70–90°.

*Methyl 3(α)-Acetoxy-12-chloro-Δ<sup>9,11</sup>-cholenate (VI) from VIII*—A solution of 4.61 gm. of methyl 3(α)-acetoxy-12-methoxy-Δ<sup>9,11</sup>-cholenate (VIII) (1) in 50 cc. of dry, alcohol-free chloroform was cooled in an ice bath and saturated with dry hydrogen chloride over a period of 35 minutes. After an additional 25 minutes the solution was concentrated under reduced pressure to a small volume. The residue, crystallized from petroleum ether, gave a 92 per cent yield of crude product which melted at 130–135°. The analytic sample, which was prepared by crystallization from ethyl acetate-ligroin, melted at 137.5–138.5°.  $[\alpha]_D = +154^\circ \pm 2^\circ$  (30.7 mg. in 3.00 cc. of chloroform).

$C_{27}H_{41}O_4Cl$ . Calculated. C 69.73, H 8.89, Cl 7.62  
Found. " 69.62, " 9.04, " 7.40

*VI Recovered from Water-Chloroform Mixture*—930 mg. of methyl 3(α)-acetoxy-12-chloro-Δ<sup>9,11</sup>-cholenate (VI), 290 mg. of silver carbonate, 20 cc. of water, 20 cc. of chloroform, and 25 gm. of glass beads were placed in a glass-stoppered bottle and rotated for 15 hours. Only 4 per cent of the theoretical weight of silver chloride was formed. The chloroform was removed under reduced pressure and 700 mg. of material were recovered by addition of petroleum ether. The melting point was 135–136° and the compound recovered did not depress the melting point of the starting material.  $[\alpha]_D = +153^\circ \pm 2^\circ$  (29.8 mg. in 3 cc. of chloroform). A second crop of 108 mg., m.p. 133–134.5°, was obtained.

*Methyl 3(α)-Acetoxy-12-hydroxy-Δ<sup>9,11</sup>-cholenate (VII) from VI*—Methyl 3(α)-acetoxy-12-chloro-Δ<sup>9,11</sup>-cholenate (VI) (930 mg.) was dissolved in 50 cc. of acetone, and 20 cc. of water, 1.10 gm. of silver carbonate, and 38 gm. of glass beads were added. The mixture was rotated in a glass-stoppered container for 3 hours and was filtered. The silver chloride obtained was 98 per cent of the theoretical amount. The filtrate was concentrated under reduced pressure to about 10 cc. and the crystals which separated were removed and washed with acetone-water 1:1. After recrystallization from chloroform-ligroin, 803 mg. of material which melted at 100–102° were obtained. The melting point of the finely ground material was 92–95°. The compound was prepared several times, but the melting point was never sharp and frequently was spread over a wide range.  $[\alpha]_D = +100^\circ \pm 2^\circ$  (29.8 mg. in 3.00 cc. of methanol).

*Methyl 3(α)-Acetoxy-12-keto-Δ<sup>9,11</sup>-cholenate (IX) from VII*—A solution of 446 mg. of methyl 3(α)-acetoxy-12-hydroxy-Δ<sup>9,11</sup>-cholenate (VII) in 10 cc. of acetic acid was mixed at room temperature with 1.66 cc. of 1.88 N chromic acid in acetic acid. After 30 minutes benzene was added and the organic phase was washed with water, with a dilute solution of sodium bicarbonate, and finally with water. The benzene solution was

filtered through sodium sulfate and concentrated to dryness under reduced pressure. The residue crystallized from cold methanol to give 353 mg. of product which melted at 145–149°. After recrystallization from methanol the material melted at 148–149° and did not depress the melting point of methyl 3( $\alpha$ )-acetoxy-12-keto- $\Delta^9,^{11}$ -cholenate (1, 13, 14, 17).  $[\alpha]_D = +103^\circ \pm 2^\circ$  (30.9 mg. in 3.00 cc. of chloroform).

*3,9-Epoxy- $\Delta^{11}$ -cholenic Acid (X) from I*—A solution of 20 cc. of 2 N hydrogen bromide in 80 per cent acetic acid-20 per cent water was added to 10.00 gm. of 3( $\alpha$ ),12-dihydroxy- $\Delta^9,^{11}$ -cholenic acid (I) (1, 2) dissolved in 250 cc. of glacial acetic acid at room temperature. After 6 minutes 1 liter of chloroform was added and the chloroform solution was washed with water until free of acetic acid. The chloroform was removed under reduced pressure. The residue was dissolved in ethanol and poured into 600 cc. of 1 N aqueous solution of sodium hydroxide. The solution was concentrated under reduced pressure until all of the ethanol had been removed and was then cooled in an ice bath. The crystals which formed were filtered and dissolved in water. The solution was acidified with acetic acid and extracted with chloroform. The chloroform solution was washed free from acetic acid with water and concentrated to dryness under reduced pressure. Crystallization of the residue from ethanol-water gave 8.21 gm. (86 per cent) of crude material which melted at 151–155°. A sample purified by crystallization from methanol-water melted at 157–158°.  $[\alpha]_D = -57^\circ \pm 2^\circ$  (37.6 mg. in 3.76 cc. of methanol). The molecular weight was determined according to the Rast method (18). Theory, 373; found, 344.

$C_{21}H_{34}O_2$ . Calculated, C 77.38, H 9.74; found, C 77.23, H 9.76

*3,9-Epoxy- $\Delta^{11}$ -cholenic Acid (X) Recovered Unchanged from Treatment with Acetic Anhydride and Pyridine*—A sample of 3,9-epoxy- $\Delta^{11}$ -cholenic acid (1.0 gm.) was dissolved in 6 cc. of pyridine and 6 cc. of acetic anhydride at room temperature. After 48 hours the addition of ice caused the separation of 967 mg. of crystals which melted at 155–156°. After recrystallization from dilute acetone the material melted at 157–158° and did not depress the melting point of 3,9-epoxy- $\Delta^{11}$ -cholenic acid.  $[\alpha]_D = -57^\circ \pm 2^\circ$  (31.1 mg. in 3.00 cc. of methanol).

*Stability of 3,9-Epoxy- $\Delta^{11}$ -cholenic Acid (X) in Methanol*— $[\alpha]_D = -58^\circ \pm 2^\circ$  (30.7 mg. in 3.00 cc. of methanol). The rotation remained constant for 48 hours.

*Methyl 3,9-Epoxy- $\Delta^{11}$ -cholenate (XI) from X*—A sample of 3,9-epoxy- $\Delta^{11}$ -cholenic acid was esterified with an excess of diazomethane in ether. The product, crystallized slowly from cold methanol and water, melted at 53–54°.  $[\alpha]_D = -59^\circ \pm 2^\circ$  (25.0 mg. in 4.00 cc. of chloroform).

$C_{25}H_{40}O_2$ . Calculated.	C 77.67, H 9.91
Found.	" 78.24, " 10.21
	" 78.08, " 10.26

*Stability of Methyl 3,9-Epoxy- $\Delta^{11}$ -cholenate (XI) in Acetic Acid*— $[\alpha]_D = -63^\circ \pm 2^\circ$  (30.5 mg. in 3.00 cc. of acetic acid). The rotation remained constant for 48 hours.

*XI from IV*—A solution of 845 mg. of methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV) in 10 cc. of chloroform was shaken with 0.5 gm. of sodium bicarbonate in 10 cc. of water for 21 hours. The halogen removed was 99 per cent of the theoretical amount. The chloroform solution was washed with water, dried with sodium sulfate, and concentrated to dryness under reduced pressure. The residue crystallized from cold acetone-water to give 676 mg. of product which melted at  $52.5$ – $53.5^\circ$  and did not depress the melting point of XI prepared from X.  $[\alpha]_D = -59^\circ \pm 2^\circ$  (29.9 mg. in 3 cc. of chloroform).

*XI from IV*—845 mg. of methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9,^{11}$ -cholenate (IV) in 10 cc. of chloroform and 2 cc. of pyridine were boiled under a reflux for 2 hours, cooled to room temperature, diluted with chloroform, and washed with water, dilute sulfuric acid, and water. The aqueous washings contained 98.5 per cent of the theoretical amount of chloride ion. The chloroform solution was dried with sodium sulfate, the bulk of the solvent was removed under reduced pressure, and the last traces were displaced with acetone. The residue crystallized from cold acetone-water to give 670 mg. of material which melted at  $53$ – $54^\circ$  and did not depress the melting point of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate prepared from X with diazomethane.  $[\alpha]_D = -59^\circ \pm 2^\circ$  (31.6 mg. in 3 cc. of chloroform).

*XI from V*—200 mg. of methyl 3( $\alpha$ )-hydroxy-12-bromo- $\Delta^9,^{11}$ -cholenate (V) (m.p.  $137$ – $138^\circ$ ) were dissolved in 10 cc. of chloroform and the solution was washed with water until the last washing did not contain bromide ion. Eight 10 cc. portions of water were used. The halogen in the aqueous phase was 98 per cent of the theoretical amount. The chloroform was removed under reduced pressure and the residue was crystallized from cold acetone-water. The product (76 mg.) melted at  $52.5$ – $53.5^\circ$  and did not depress the melting point of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate which was made from X with diazomethane.  $[\alpha]_D = -58^\circ \pm 2^\circ$  (31.7 mg. in 3 cc. of chloroform).

*XI from XII*—16.4 gm. of methyl 3,9-epoxy-11,12-dibromocholanate (XII) were suspended in 200 cc. of methanol and heated almost to boiling. 15.0 gm. of zinc dust were added in small portions over an interval of 5 minutes. The solution was heated to boiling under a reflux for 40 minutes, the excess of zinc was filtered from the solution, and the methanol was removed under reduced pressure. The residue was distributed between



water and benzene and the organic phase was washed with water, dried with sodium sulfate, and concentrated under reduced pressure. The last traces of benzene were displaced with acetone. Addition of water to the acetone solution cooled in ice caused the separation of 9.33 gm. of material which melted at 51–52°. Further dilution with water yielded a second crop of 1.17 gm. which melted at 48–50°.  $[\alpha]_D = -52^\circ \pm 2^\circ$  (100.4 mg. of Crop 1 made to 10 cc. in methanol);  $[\alpha]_D = -59^\circ \pm 2^\circ$  (101.1 mg. made to 10 cc. in chloroform).

*XI from XIII*—30.0 gm. of methyl 3,9-epoxy-11,12-dibromocholanoate (XIII) were treated with 30.0 gm. of zinc dust in 350 cc. of methanol in the manner described under "XI from XII." Dilution of the ice-cold acetone solution with water gave four crops of crystals: 11.60 gm., m.p. 52–53°; 5.92 gm., m.p. 53–54°; 1.73 gm., m.p. 53–54°; and 0.320 gm., m.p. 49–53°.  $[\alpha]_D = -58^\circ \pm 2^\circ$  (109.7 mg. of Crop 1 made to 10 cc. in chloroform).

*Methyl 3,9-Epoxy-11,12-dibromocholanoate (XII) from XI*—126 gm. of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9,^{11}$ -cholenate were converted to methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (XI) with hydrochloric acid and pyridine as described under "IV from II" and "XI from IV." Without isolation of the two intermediate products the crude compound in 300 cc. of dry, alcohol-free chloroform was cooled in a dry ice-acetone bath and added to a solution of 31 cc. of bromine in 360 cc. of chloroform (also cooled in a dry ice-acetone bath) in three portions at 1 minute intervals. The solutions were protected from moisture with calcium chloride tubes and after 30 minutes the excess bromine was removed by pouring the chloroform solution into 2 liters of water which contained 53 gm. of sodium bicarbonate and 53 gm. of sodium sulfite. The mixture was stirred for 30 minutes. The organic phase was separated, washed with water, and filtered through anhydrous sodium sulfate. Titration of the aqueous phase indicated that 100.5 per cent of the theoretical amount of bromine had been consumed. The filtrate was concentrated under reduced pressure to 235 cc., the temperature was adjusted to 25°, and 750 cc. of methanol were added slowly while the flask was shaken. After 20 minutes the solution was filtered and the crystals were washed with methanol. 101.4 gm. of material which melted at 142.5–143° were obtained.  $[\alpha]_D = +45^\circ \pm 2^\circ$  (40.0 mg. in 4.00 cc. of chloroform).

$C_{25}H_{44}O_2Br_2$ . Calculated, C 54.95, H 7.01; found, C 54.77, H 7.11

*XII Recovered from Excess Chromic Acid*—546 mg. (0.0010 mole) of methyl 3,9-epoxy-11,12-dibromocholanoate (XII) were dissolved in 10 cc. of chloroform and 12 cc. of acetic acid which contained 4 cc. of 1 N chromic acid. After 2 hours chloroform and water were added and the organic phase was washed with water, with dilute sodium carbonate solution,

and finally with water. The aqueous phase contained 2.7 cc. of 1 N chromic acid. The chloroform solution was dried and concentrated to dryness under reduced pressure. The residue crystallized from ether-methanol to give 336 mg. of material which melted at 138–141°. After recrystallization from ether-methanol the product melted at 142.5–143° and did not depress the melting point of XII.

*Methyl 3,9-Epoxy-11,12-dibromocholanate (XIII) from XI*—15.8 gm. of material which crystallized from the mother liquor after separation of the dibromide (XII) (see "XII from XI") melted at 115–120° and a final crop, after concentration of the solution (21.1 gm.), melted at 98–108°. After several recrystallizations of the combined crops from ether-methanol the product melted at 123–123.5°.  $[\alpha]_D = +20^\circ \pm 2^\circ$  (31.1 mg. in 3.00 cc. of chloroform).

$C_{25}H_{41}O_2Br_2$ . Calculated, C 54.95, H 7.01; found, C 55.11, H 7.16

*3,9-11,12-Diepoxycholanolic Acid (XIV) from X*—2.88 cc. of 0.80 N perbenzoic acid (1.15 moles) in chloroform were added to 370 mg. of 3,9-epoxy- $\Delta^{11}$ -cholenic acid (X) in 2 cc. of chloroform at 0°. After 24 hours at 5° 96 per cent of 1 mole of perbenzoic acid had been consumed. The chloroform solution was washed with a dilute solution of potassium iodide and sodium thiosulfate, and then with water. The solvent was removed under reduced pressure and the residue was separated in two crops from ether-ligroin. 208 mg. melted at 173–174°; 26 mg. melted at 165–168°. A sample purified by crystallization from ether-ligroin melted at 177–177.5°.  $[\alpha]_D = +23^\circ \pm 2^\circ$  (30.7 mg. in 3.00 cc. of methanol);  $[\alpha]_D = +14^\circ \pm 2^\circ$  (32.2 mg. in 3.00 cc. of chloroform).

$C_{24}H_{38}O_4$ . Calculated, C 74.19, H 9.34

Found. " 74.52, " 9.36

" 74.57, " 9.48

For comparison with the epimeric 11,12-epoxide see "XXVII from XXVI."

*Methyl 3,9-11,12-Diepoxycholanate (XV) from XIV*—220 mg. of 3,9-11,12-diepoxycholanolic acid (XIV) were esterified with a solution of diazomethane in ether. The ester which crystallized from dilute methanol weighed 203 mg. and melted at 92.5–93.5°.  $[\alpha]_D = +16^\circ \pm 2^\circ$  (30.4 mg. in 3.00 cc. of chloroform).

$C_{25}H_{38}O_4$ . Calculated, C 74.59, H 9.51; found, C 74.68, H 9.60

For comparison with the methyl ester of the epimeric 11,12-epoxide see "XXVIII from XXVII."

*Methyl 3( $\alpha$ )-Hydroxy-12-acetoxy- $\Delta^9$ ,  $\Delta^{11}$ -cholenate (XVI) (I) from XI*—772 mg. of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (XI) were dissolved in 20 cc. of

acetic acid and 0.66 cc. of 5 N aqueous sulfuric acid was added at room temperature. After 2 hours the reaction mixture was distributed between water and benzene and the organic phase was washed with water, with dilute sodium carbonate solution, and finally with water. The solvent was removed under reduced pressure and the residue was crystallized from ligroin to give 308 mg. of material which melted at 107–108° and did not depress the melting point of XVI prepared from II (1).  $[\alpha]_D = +195^\circ \pm 2^\circ$  (30.0 mg. in 3 cc. of chloroform).

*3( $\alpha$ )-Hydroxy- $\Delta^9$ , $^{11}$ -cholenic Acid (XVII) from X*—7.44 gm. of 3,9-epoxy- $\Delta^{11}$ -cholenic acid (X) in 75 cc. of acetic acid were reduced with hydrogen in the presence of 100 mg. of Adams' platinum oxide catalyst. After 10 minutes 101 per cent and after 16 minutes 110 per cent of 1 molar equivalent of hydrogen had been utilized. The platinum was filtered from solution and 100 cc. of water were added to the acetic acid solution. The product which separated (7.29 gm.) was washed with 50 per cent acetic acid, dried, and dissolved in 200 cc. of a hot 1:1 mixture of acetone and benzene. 5.64 gm. of crystalline material separated after the solution had stood at 5° for several hours. The melting point was 187.5–188.5°.  $[\alpha]_D = +45^\circ \pm 2^\circ$  (30.6 mg. in 3 cc. of methanol).

The cholenic acid XVII has been repeatedly studied. It has been encountered as the reduction product of II, III, XI, and XVI. Prepared from II,

$C_{27}H_{44}O_2$ .	Calculated.	C 76.91, H 10.23
	Found.	" 76.93, " 10.54
		" 76.63, " 10.32

Five derivatives of XVII have been made: lithocholic acid and its methyl ester and compounds XVIII, XIX, and XX, which are described in the following paragraphs. The methyl ester XVIII was hydrolyzed to give XVII which melted at 190–191° (13, 14).

*Lithocholic Acid and Methyl Lithocholate from 3( $\alpha$ )-Hydroxy- $\Delta^9$ , $^{11}$ -cholenic Acid (XVII)*—500 mg. of 3( $\alpha$ )-hydroxy- $\Delta^9$ , $^{11}$ -cholenic acid (XVII) in 20 cc. of glacial acetic acid were shaken in an atmosphere of hydrogen with 100 mg. of Adams' platinum oxide catalyst for 18 hours. The platinum was filtered off. 100 mg. of fresh catalyst were added and the reduction was continued for 6.5 hours. The platinum was once more replaced with 100 mg. of fresh catalyst and the reduction was continued for 14 hours. The platinum was filtered from solution. The filtrate, diluted with water, gave 480 mg. of material which melted at 174–178°. Two recrystallizations yielded 247 mg. of crystals which melted at 185–187°. The acid was esterified with diazomethane; 202 mg. of ester were obtained by crystallization from ligroin. After several crystallizations from dilute methanol the ester melted at 128–129° and did not depress the melting

point of methyl lithocholate (12, 15, 16).  $[\alpha]_D = +35^\circ \pm 2^\circ$  (25.7 mg. in 3.00 cc. of methanol).

$C_{25}H_{42}O_3$ . Calculated, C 76.87, H 10.84; found, C 77.01, H 11.26

*Methyl 3( $\alpha$ )-Hydroxy- $\Delta^9,^{11}$ -cholenate (XVIII) from XVII*—422 mg. of 3( $\alpha$ )-hydroxy- $\Delta^9,^{11}$ -cholenic acid (XVII) were esterified with an ether solution of diazomethane. The ester which crystallized from methanol and water (404 mg.) melted at 102–103°. After recrystallization from methanol

TABLE I  
Production of Compound XVIII from Compound XI

Experiment No.	Lithocholic acid	Ethylmorpholine	Time	Per cent of 1 mole of $H_2$
	<i>mM</i>	<i>mM</i>		
1	0	0	2 hrs., 35 min.	15
2*	0	0	57 "	10
3†	0	0	1 hr., 48 "	5
4	0	1.0	1 "	0
5	1.0	0	12 hrs.	91
6	0.5	0	22 " 15 min.	98
7	1.0	1.0	4 "	2
8	1.0	1.0	2 " 50 min.	0
9	0	0	1 hr., 53 "	9
9A‡	0	0	15 hrs.	105
10§	0	0	1 hr., 23 min.	0
10A	0.5	0	69 hrs., 30 "	83

\* Absolute alcohol free of aldehyde.

† 85 per cent ethanol free of aldehyde.

‡ 1 cc. of acetic acid was added to Experiment 9 and the reduction was continued (Experiment 9A).

§  $PtO_2$  was reduced in 20 cc. of absolute ethanol. 386 mg. of XI were then added.

|| After 1 hour, 23 minutes, during which the ester XI was not reduced, 0.5 equivalent of lithocholic acid was added.

the melting point was 105–105.5°. The melt recrystallized on the cover-glass when scratched and melted again at 112–113°. When the melted sample was allowed to cool to 108° and was scratched, the crystals which formed melted at 112–113°. When the sample was allowed to cool to less than 100° and was then scratched, the crystals which formed melted at 105–105.5° (14).  $[\alpha]_D = +46^\circ \pm 2^\circ$  (29.7 mg. in 3.00 cc. of methanol).

$C_{25}H_{40}O_3$ . Calculated, C 77.27, H 10.38; found, C 77.48, H 10.42

*Catalytic Reduction of Methyl 3,9-Epoxy- $\Delta^{11}$ -cholenate (XI)*—In the following experiments 0.0010 mole (386 mg.) of XI was dissolved in approximately 20 cc. of 95 per cent ethanol, aldehyde-free, in an all-glass container

which permitted vigorous agitation. Basic and acidic mediums were provided by the addition of ethylmorpholine, acetic acid, or lithocholic acid in the amounts indicated in Table I. The time required for the reduction is recorded in the next to the last column. In each experiment of less than 3 hours duration, the uptake of hydrogen had stopped at least 15 minutes before the experiment was terminated.

*Catalytic Reduction of 3,9-Epoxy- $\Delta^{11}$ -cholenic Acid (X)*—Under conditions identical with those described under catalytic reduction of XI in the preceding paragraph the epoxy acid X was treated in 95 per cent ethanol, aldehyde-free. The results are shown in Table II.

*Methyl 3( $\alpha$ )-Hydroxy- $\Delta^{9,11}$ -cholenate (XVIII) from XI*—3.86 gm. of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate were reduced with hydrogen and 200 mg. of Adams' platinum oxide in a solution of 50 cc. of ethanol which contained 1 cc. of acetic acid (see Table I). 102 per cent of the theoretical amount

TABLE II  
Effect of Ethylmorpholine on Utilization of Hydrogen

Experiment No.	Ethylmorpholine	Time	Per cent of 1 mole of H <sub>2</sub>
	<i>mM</i>		
1	0	4 hrs., 6 min.	86
2	2.0	16 " 10 "	0
3*	1.0	15 "	87

\* Instead of compound X, methyl 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholenate (12, 19) was used to show that ethylmorpholine did not poison the platinum catalyst and did not prevent absorption of hydrogen by the double bond C<sub>11</sub>:C<sub>12</sub>.

of hydrogen was absorbed in 7 minutes. After removal of the platinum  $[\alpha]_D = +44^\circ \pm 2^\circ$  ( $\alpha = +2.9^\circ$ ; volume 59 cc.) for the reduced material.  $[\alpha]_D$  of XVII =  $+46^\circ \pm 2^\circ$  (30.1 mg. in 3 cc. of ethanol which contained 2 per cent of acetic acid).

*Methyl 3( $\alpha$ )-Acetoxy- $\Delta^{9,11}$ -cholenate (XIX) from XVIII*—The acetoxy derivative of XVIII is more easily purified than is the 3( $\alpha$ )-hydroxy compound and for separation of the reduced material obtained in the preceding paragraph the product was acetylated. The solvents were removed under reduced pressure and the last traces were displaced with a small amount of acetic anhydride which was also removed. The residue was dissolved in 5 cc. of acetic anhydride and 5 cc. of pyridine at room temperature. After 18 hours benzene was added and the organic phase was washed with dilute hydrochloric acid and with water. The benzene was removed under reduced pressure and the residue was dissolved in 100 cc. of boiling methanol. After the solution was cooled in ice, 3.46 gm. of crystals were separated, m.p. 135–136°. Recrystallization gave 3.1 gm. of material which melted at

136.6–137°. When a sample was mixed with a sample of methyl 3( $\alpha$ )-acetoxy- $\Delta^9,11$ -cholenate, the melting point was 136.6–137.5° (14).  $[\alpha]_D = +56^\circ \pm 2^\circ$  (100.9 mg. in acetone; total volume 10 cc.).

*Methyl 3( $\alpha$ )-Acetoxy- $\Delta^9,11$ -cholenate (XIX) (14) from XVII*—5.0 gm. of 3( $\alpha$ )-hydroxy- $\Delta^9,11$ -cholenic acid (XVII) were dissolved in 250 cc. of 0.5 N methanolic hydrogen chloride. After 18 hours at room temperature the solvent was removed under reduced pressure, the residue was dissolved in benzene, and the solution was washed with water. The benzene was removed under reduced pressure and the residue was dissolved in 10 cc. of benzene, 25 cc. of pyridine, and 25 cc. of acetic anhydride at room temperature. After 18 hours benzene was added and the organic phase was washed with water, with dilute hydrochloric acid, with a dilute sodium bicarbonate solution, and finally with water. The solvent was removed under reduced pressure and the residue, crystallized from acetone, gave 4.93 gm. of material which melted at 135–136°. After recrystallization from methanol the product melted at 136.5–137.5°.  $[\alpha]_D = +57^\circ \pm 2^\circ$  (20.0 mg. in 4.00 cc. of chloroform).

$C_{27}H_{42}O_4$ . Calculated, C 75.30, H 9.83; found, C 75.24, H 9.70

*3( $\alpha$ )-Hydroxy-9,11-epoxycholenic Acid (XX) from XVII*—2.77 gm. of 3( $\alpha$ )-hydroxy- $\Delta^9,11$ -cholenic acid (XVII) were added to 116 cc. of chloroform which contained 1.43 molar equivalents of perbenzoic acid. After 14 hours at 5° the perbenzoic acid consumed was 78 per cent of the theoretical amount. The solution was washed with a dilute solution of potassium iodide and sodium thiosulfate, and with water. The chloroform was evaporated to dryness under reduced pressure. After two recrystallizations from dilute methanol the product (819 mg.) melted at 165–167°. After repeated recrystallizations from benzene, a product was obtained which melted at 199.5–200.5°.  $[\alpha]_D = +36^\circ \pm 2^\circ$  (32.9 mg. in 3.00 cc. of methanol).

$C_{27}H_{42}O_4$ . Calculated. C 73.81, H 9.81  
 Found. " 74.33, " 9.88  
 " 74.27, " 10.11

*Methyl 3,12-Diketo- $\Delta^9,11$ -cholenate (XXI) from X*—1.0 gm. of 3,9-epoxy- $\Delta^{11}$ -cholenic acid (X) in 5 cc. of chloroform and 5 cc. of acetic acid was treated with 12 cc. of 2 N chromic acid for 30 minutes in an ice bath and for 5 hours at room temperature. Chloroform was added and the organic phase was washed with water and concentrated to a dry residue under reduced pressure. The material was treated with diazomethane in ether and the solution was washed with dilute hydrochloric acid and with water. The ether was removed under reduced pressure and the residue was dissolved in 100 cc. of a mixture of benzene-petroleum ether (2:1) and

fractionated on a column of 30 gm. of aluminum oxide. Elution of fractions with mixtures of benzene and petroleum ether and later with benzene removed 392 mg., which were not identified. Further elution with benzene and with benzene which contained 0.10 per cent methanol removed 370 mg. of material from which 93 mg. were separated by crystallization from ligroin. The product melted at 128–129.5° and did not depress the melting point of methyl 3,12-diketo- $\Delta^9$ ,<sup>11</sup>-cholenate (1, 2, 14).  $[\alpha]_D = +63^\circ \pm 2^\circ$  (27.5 mg. in 4 cc. of chloroform). For XXI from II see Paper III of this series (1).

*Methyl 3-Keto-12-hydroxy- $\Delta^9$ ,<sup>11</sup>-cholenate (XXIII) from XXII*—3.73 gm. of 3-keto-12-hydroxy- $\Delta^9$ ,<sup>11</sup>-cholenic acid (XXII) (1) were esterified with a solution of diazomethane in ether which contained a few drops of methanol. The ether solution was washed with dilute acetic acid, with dilute potassium hydroxide solution, and with water. The ether was removed under reduced pressure and the residue was crystallized from ligroin. 3.5 gm. of material melted at 157–159°; 0.60 gm. melted at 154–157°. After several recrystallizations from benzene-petroleum ether the ester melted at 161–161.5°.  $[\alpha]_D = +70^\circ \pm 2^\circ$  (31.6 mg. in 3.00 cc. of chloroform).

$C_{25}H_{44}O_4$ . Calculated, C 74.59, H 9.51; found, C 74.40, H 9.21

*XXIII from XXIV*—465 mg. of methyl 3-keto-12-bromo- $\Delta^9$ ,<sup>11</sup>-cholenate (XXIV) in 20 cc. of acetone were diluted with 20 cc. of 0.1 N aqueous solution of silver nitrate at room temperature. After 2 hours the silver bromide which separated (99 per cent of the theoretical amount) was removed by filtration and the acetone was removed under reduced pressure. The water-insoluble residue was dissolved in benzene, the organic phase was washed with water, and the solvent was removed under reduced pressure. Addition of a small volume of ligroin gave 365 mg. (90.5 per cent) of crystals which melted at 156.6–157.5° and did not depress the melting point of methyl 3-keto-12-hydroxy- $\Delta^9$ ,<sup>11</sup>-cholenate (1).  $[\alpha]_D = +70^\circ \pm 2^\circ$  (30.1 mg. in 3 cc. of chloroform).

*Methyl 3-Keto-12-bromo- $\Delta^9$ ,<sup>11</sup>-cholenate (XXIV) from XXIII*—A stream of dry hydrogen bromide was passed into an ice-cold solution of 604 mg. of methyl 3-keto-12-hydroxy- $\Delta^9$ ,<sup>11</sup>-cholenate (XXIII) in 25 cc. of dry, alcohol-free chloroform during 15 minutes. After an additional 15 minutes, the chloroform solution was washed with water, dried with sodium sulfate, and concentrated under reduced pressure almost to dryness. On addition of a small volume of ligroin 513 mg. of crystals formed which melted at 137–138°.  $[\alpha]_D = +182^\circ \pm 2^\circ$  (31.9 mg. in 3.00 cc. of chloroform). The compound decomposed on long standing at room temperature. For analysis see the following paragraph.

*XXIV from XXV*—Dry hydrogen bromide was passed into an ice-cold solution of 402 mg. of methyl 3-keto-12-methoxy- $\Delta^9,^{11}$ -cholenate (XXV) (1) in 50 cc. of chloroform for about 20 minutes. The solution was concentrated under reduced pressure to a small volume and diluted with petroleum ether. 370 mg. of material separated which melted at 129–132°; 20 mg. melted at 132.5–134°. Repeated recrystallization from benzene-petroleum ether gave needles which melted at 136–137°.  $[\alpha]_D = +182^\circ \pm 2^\circ$  (31.9 mg. in 3.00 cc. of chloroform).

$C_{28}H_{46}O_4Br$ . Calculated, C 64.50, H 8.01; found, C 64.60, H 8.09

*XXIV Recovered from Methanol after Heating at Boiling Point*—200 mg. of methyl 3-keto-12-bromo- $\Delta^9,^{11}$ -cholenate (XXIV) were dissolved in 10 cc. of boiling methanol and the solution was refluxed for 1 minute. The solvent was removed under reduced pressure and the residue was crystallized from ice-cold petroleum ether. 109 mg. of material melted at 127–129°. After two recrystallizations from ether-petroleum ether the product (52 mg.) melted at 135–136° and did not depress the melting point of methyl 3-keto-12-bromo- $\Delta^9,^{11}$ -cholenate.  $[\alpha]_D = +183^\circ \pm 2^\circ$  (30.2 mg. in 3.00 cc. of chloroform).

Although XXIV is stable for a short interval at the boiling point of methanol, the bromine is slowly replaced with methoxyl even at 0°. 40 per cent of the bromine was found as hydrobromic acid 23 hours after XXIV was dissolved in methanol and held at 0°.

*XXIV Recovered from Hot Water-Chloroform*—15 cc. of water and 15 cc. of chloroform which contained 150 mg. of methyl 3-keto-12-bromo- $\Delta^9,^{11}$ -cholenate (XXIV) were heated to boiling under a reflux for 1 hour. The aqueous phase gave only a slight turbidity with silver nitrate.

*Methyl 3-Keto-12-methoxy- $\Delta^9,^{11}$ -cholenate (XXV) from XXIV*—200 mg. of methyl 3-keto-12-bromo- $\Delta^9,^{11}$ -cholenate (XXIV) were dissolved in a solution of 170 mg. of silver nitrate in 20 cc. of methanol at room temperature. After 30 minutes, the silver bromide (80 mg., which was 99 per cent of the theoretical amount) was removed and the methanol solution was concentrated to a small volume in an air current and diluted with water. Crystals formed slowly. After recrystallization from dilute methanol the product (50 mg.) melted at 97–98° and did not depress the melting point of methyl 3-keto-12-methoxy- $\Delta^9,^{11}$ -cholenate (1).

*Methyl 3,9-Epoxy-11-acetoxy-12-bromocholanate (XXVI)<sup>9</sup> from XII*—197 gm. of methyl 3,9-epoxy-11,12-dibromocholanate (XII) and 90 gm. of sodium acetate were dissolved in 3 liters of glacial acetic acid and the solution was refluxed for 3 hours. The solvent was removed under reduced

<sup>9</sup> The reason for the formulation with the acetoxyl group at  $C_{11}$  will be presented in Paper V of this series.



pressure and the residue was distributed between benzene and water. The organic phase was washed with water, with a sodium bicarbonate solution, and again with water, and was dried with sodium sulfate. The aqueous phase contained the theoretical amount of bromide ion for 1 equivalent. The benzene was concentrated under reduced pressure to a small volume and the remaining solvent was displaced with acetone. The residue was crystallized from acetone-water and gave 159 gm. of material which melted at  $133.5\text{--}134^\circ$ , and 12.5 gm. which melted at  $131.5\text{--}132.5^\circ$ .  $[\alpha]_D = +17^\circ \pm 2^\circ$  (20.0 mg. in 4.00 cc. of chloroform).

$C_{21}H_{30}O_4Br$ . Calculated, C 61.70, H 7.86; found, C 61.83, H 7.93

*3,9-11,12-Diepoxycholanolic Acid (XXVII) from XXVI*—171.5 gm. of methyl 3,9-epoxy-11-acetoxy-12-bromocholanate (XXVI) were added to 3250 cc. of 1 N methanolic sodium hydroxide and the solution was boiled under a reflux for 3 hours. The methanol was removed under reduced pressure until crystals formed. About 3 liters of water were added and the solution was concentrated until crystals formed. About 2.5 liters of benzene were added, and the solution was acidified with acetic acid and separated into two phases. The aqueous phase was extracted with benzene. The combined benzene extracts were washed with water, dried with sodium sulfate, and concentrated under reduced pressure to a small volume. The residue, crystallized from acetone, gave 100.0 gm. of material which melted at  $181\text{--}182^\circ$ . Two additional crops were obtained (18.4 gm., m.p.  $180\text{--}181^\circ$ ; 6.7 gm., m.p.  $179\text{--}180^\circ$ ) by dilution of the filtrate with water, to bring the total yield to 99 per cent of the theoretical amount.  $[\alpha]_D = +14^\circ \pm 2^\circ$  (20.0 mg. in 4 cc. of chloroform).

$C_{21}H_{30}O_4$ . Calculated, C 74.19, H 9.34; found, C 74.48, H 9.20

The melting point of a mixture of XXVII and its epimer XIV was depressed to  $170\text{--}173^\circ$ .

*Methyl 3,9-11,12-Diepoxycholanate (XXVIII) from XXVII*—388 mg. of 3,9-11,12-diepoxycholanolic acid (XXVII) were esterified with diazomethane in ether. The ester, crystallized from methanol-water, weighed 350 mg., and melted at  $80\text{--}81^\circ$ .  $[\alpha]_D = +16^\circ \pm 2^\circ$  (31.9 mg. in 3.00 cc. of chloroform).

$C_{22}H_{32}O_4$ . Calculated, C 74.59, H 9.51; found, C 74.75, H 9.33

Contrary to the effect on the melting point when the epimeric acids XIV and XXVII were mixed, the melting point of XXVIII when mixed with XV was not depressed.

*3,9-Epoxy-11,12-dihydroxycholanolic Acid (M.p.  $240^\circ$ ) (XXIX) from XXVII*—62 gm. of 3,9-11,12-diepoxycholanolic acid (XXVII) were

dissolved in 1240 cc. of glacial acetic acid which was 0.01 N with sulfuric acid. After 85 minutes at room temperature 2 gm. of sodium acetate were added and the solvent was removed under reduced pressure. The residue was distributed between benzene and water and the organic phase was washed with water to remove the acetic acid. The bulk of the benzene was removed under reduced pressure and the remainder was displaced with methanol. The residue was dissolved in 300 cc. of methanol, 760 cc. of 2.5 N aqueous solution of sodium hydroxide were added, and the solution was refluxed for 15 hours. The solution was concentrated under reduced pressure to about 500 cc. to remove the methanol. About 2.5 liters of water and 500 cc. of benzene were added, and while being stirred mechanically the solution was acidified with 130 cc. of acetic acid. The crystals which formed were collected and washed with water and benzene. 27.55 gm. of material (42.5 per cent) which melted at 229–232° were obtained. A sample prepared by recrystallization from acetone-water melted at 239–240°.  $[\alpha]_D = +59^\circ \pm 2^\circ$  (33.5 mg. in 4.00 cc. of methanol).

$C_{24}H_{38}O_5$ . Calculated, C 70.90, H 9.42; found, C 70.84, H 9.40

*Methyl 3,9-Epoxy-11,12-dihydroxycholanate (XXX) from XXIX*—406 mg. of 3,9-epoxy-11,12-dihydroxycholanic acid (XXIX) were esterified with a solution of diazomethane in ether and the ester was crystallized from methanol-water. 400 mg. which melted at 104–105° were obtained.  $[\alpha]_D = +59^\circ \pm 2^\circ$  (31.5 mg. in 3.00 cc. of methanol).

$C_{26}H_{40}O_6$ . Calculated, C 71.39, H 9.59; found, C 71.34, H 9.76

*3,9-Epoxy-11,12-dihydroxycholanic Acid (M.p. 149°) (XXXI) from XXVII*—Separation of the glycol (m.p. 149°) was accomplished by concentration of the benzene solution described under "XXIX from XXVII" from which 27.55 gm. of glycol XXIX had been separated. Petroleum ether was added to the benzene solution at a volume of 80 cc. 26.65 gm. (41 per cent) of material which melted at 143–145° were obtained. Recrystallization from ethyl acetate-petroleum ether raised the melting point to 149–149.5°.  $[\alpha]_D = +49^\circ \pm 2^\circ$  (32.2 mg. in 3.00 cc. of methanol).

$C_{24}H_{38}O_5$ . Calculated, C 70.90, H 9.42; found, C 70.58, H 9.13

*3,9-Epoxy-11,12-diketocholanic Acid (XXXII) from XXIX*—149.0 cc. of 1.88 N chromic acid in 95 per cent acetic acid were added dropwise over a period of 45 minutes to 27.2 gm. of 3,9-epoxy-11,12-dihydroxycholanic acid (XXIX) in 1500 cc. of acetic acid. The solution was maintained at 18–20° and stirred for about 3.5 hours. A slight excess of sodium sulfite was added and the solvent was removed under reduced pressure. The residue was distributed between benzene and water and the benzene solu-

tion was washed with water, filtered through sodium sulfate, and concentrated to dryness under reduced pressure. The residue crystallized from dilute methanol to give 14.4 gm. of product which melted at 168–170°. The 14.4 gm. of acid were reoxidized in a similar manner in 337 cc. of acetic acid with 19 cc. of 1.88 N chromic acid in 95 per cent acetic acid. After crystallization from methanol-water the product (11.03 gm.) melted at 191–193°. The 11.03 gm. of acid were again oxidized in 260 cc. of acetic acid with 14.6 cc. of 1.88 N chromic acid in 95 per cent acetic acid. 9.44 gm. of crystalline material which melted at 203–204.5° were obtained.<sup>10</sup> When the mother liquors were combined and reoxidized, the over-all yield was raised to 41 per cent. After several recrystallizations from methanol-water the compound melted at 204.5–205°.  $[\alpha]_D = +189^\circ \pm 2^\circ$  (31.8 mg. in 3.00 cc. of methanol).

$C_{21}H_{34}O_8$ . Calculated, C 71.61, H 8.51; found, C 71.59, H 8.54

XXXII from XXXI—2.9 gm. of 3,9-epoxy-11,12-dihydroxycholelanic acid (XXXI) were oxidized in a manner similar to that described under "XXXII from XXIX." 0.56 gm. (27 per cent yield) of product was obtained. The product melted at 203.5–204° and did not depress the melting point of 3,9-epoxy-11,12-diketocholelanic acid which was prepared from the higher melting glycol (XXIX).  $[\alpha]_D = +184^\circ \pm 2^\circ$  (30.4 mg. in 3.00 cc. of methanol).

Methyl 3,9-Epoxy-11,12-diketocholelanate (XXXIII) from XXXII—403 mg. of 3,9-epoxy-11,12-diketocholelanic acid (XXXII) were esterified with a solution of diazomethane in ether; 370 mg. of ester were crystallized from methanol-water. The analytic sample melted at 115–115.5° and was pale yellow.  $[\alpha]_D = +184^\circ \pm 2^\circ$  (30.2 mg. in 3.00 cc. of methanol).

$C_{21}H_{34}O_8$ . Calculated, C 72.08, H 8.71; found, C 71.72, H 8.45

*Effect of Bromine on Derivatives of Methyl  $\Delta^8,^{11}$ -Cholenate and Methyl  $\Delta^{11}$ -Cholenate*—The compounds were dissolved in chloroform at  $-50^\circ$ . The volume varied between 20 and 40 cc., depending on the weight of material. An excess of powdered zinc acetate was added and the chloroform solution was stirred mechanically. 2 molar equivalents of bromine dissolved in chloroform and cooled to  $-50^\circ$  were added in one portion. Utilization of bromine was followed by titration of the unused halogen at intervals for 30 minutes. Alcohol saturated with sulfur dioxide was added to reduce the remaining bromine. The chloroform solution was washed with water and then placed in a glass-stoppered container with an equal volume of sodium bicarbonate solution. The mixture was rotated for

<sup>10</sup> It was found that the most satisfactory way to purify the diketone was through repeated oxidations.

about 18 hours and the halogen in the aqueous phase was determined. Table III shows the bromine absorbed, the total halogen ion, which is the sum of the amount in the water and that removed by the sodium bicarbonate solution, and the possible yield of a dibromo compound expressed in percentage. The position of the double bond is known in the two compounds with a ketone group at C<sub>12</sub>; the absorption spectrum with a maximum at 240 m $\mu$  shows the presence of an  $\alpha,\beta$ -unsaturated ketone. It is interesting to note that treatment of these compounds with the double bond

TABLE III

*Addition of Bromine to Derivatives of Methyl  $\Delta^{11}$ - and of  $\Delta^9,^{11}$ -Cholenate*

Compound		Compound No.*	Amount of compound used	Bromine added, 1.0 N	Bromine utilized after 3 min., 1.0 N	Bromine utilized after 30 min., 1.0 N	Bromine ion in aqueous phase, 1.0 N	Organic bromide, 1.0 N	Possible yield for dibromo compound
			mm	cc.	cc.	cc.	cc.	cc.	per cent
Methyl $\Delta^{11}$ -cholenate	3( $\alpha$ )-Hydroxy	(11, 19)	2.5	10.2	5.1	5.4	5.8	4.4	88
	3,9-Epoxy	XI	2.5	10.2	2.9	5.0	5.3	4.9	98
Methyl 3( $\alpha$ )-hydroxy- $\Delta^9,^{11}$ -cholenate; substituent at C <sub>12</sub>	C=O	(1, 14)	10.0	40.0	0.5	1.5	39.0	1.0	5
	C=O†	IX	10.0	39.3	0.3	0.8	38.4	0.9	5
	CH <sub>2</sub>	XVIII	5.0	19.5	17.7	18.2	15.6	3.9	39‡
	CHOH	III	2.5	10.0	6.9	6.9	9.6	0.4	8
	CHOCH <sub>3</sub>	II	10.0	40.0	22.5	22.5	38.4	1.6	8
	CHOAc	XVI	2.5	10.1	2.7	2.7	9.5	0.6	12
	CHCl	IV	2.0	8.0	3.0	5.0	9.3	0.7	12

\* The figures in parentheses denote bibliographic references.

† 3( $\alpha$ )-Acetoxy.

‡ Although the indicated yield of a dibromo derivative is 39 per cent, the high utilization of bromine (91 per cent of 2 moles) shows that bromine caused a deep seated change and did not form a stable dibromo addition product.

known to be C<sub>9</sub>:C<sub>11</sub> simulates the treatment with bromine of the other compounds in the series.

In addition to the determination of the halogen removed by aqueous bicarbonate, the chloroform was removed and the residue was dissolved in methanol. All derivatives of  $\Delta^9,^{11}$ -cholenic acid rapidly decomposed to give brown tarry products. The two derivatives of  $\Delta^{11}$ -cholenic acid gave crystalline dibromo addition products which were stable under the conditions of the experiment.

#### SUMMARY

1. Derivatives of 3( $\alpha$ )-hydroxy- $\Delta^9,^{11}$ -cholenic acid with C<sub>12</sub>-hydroxyl, methoxyl, or acetoxyl groups are readily converted into C<sub>12</sub>-halo compounds

by treatment with halogen acid. These compounds exhibit the usual properties of allylic halides. In strong sodium hydroxide and in aqueous solution of acetone in the presence of silver salts the halogen at  $C_{12}$  is replaced to give the 3( $\alpha$ ),12-dihydroxy compound.

2. In anhydrous non-polar solvents the halogen at  $C_{12}$  is stable but in the presence of water or pyridine halogen acid is removed and a six-membered cyclic ether between carbons 3 and 9 is formed, with a shift of the double bond to  $C_{11}:C_{12}$ .

3. Halogen acid opens the 3,9-epoxide to give the 3( $\alpha$ )-hydroxy-12-halo- $\Delta^9$ ,<sup>11</sup> compounds; with methanol and a trace of mineral acid the 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9$ ,<sup>11</sup> structure is obtained, and in acetic acid a trace of mineral acid gives the 3( $\alpha$ )-hydroxy-12-acetoxy- $\Delta^9$ ,<sup>11</sup> compound. The presence of the double bond  $C_{11}:C_{12}$  is shown by formation of an oxide and dibromo derivatives.

4. From methyl 3,9-epoxy- $\Delta^{11}$ -cholenate two epimeric 11,12-oxides have been prepared. One of these oxides is easily opened in acetic acid with a trace of mineral acid to give two of the four possible 3,9-epoxy-11,12-glycols. With chromic acid both of these glycols give the 3,9-epoxy-11,12-diketone.

5. Derivatives of  $\Delta^9$ ,<sup>11</sup>-cholenic acid with halogen at  $C_{12}$  and an acyloxy group or a ketone at  $C_3$  do not form the 3,9-epoxy structure. Removal of halogen acid results in formation of a diene.

6. Catalytic reduction of 3,9-epoxy- $\Delta^{11}$ -cholenic acid with hydrogen and platinum gives 3( $\alpha$ )-hydroxy- $\Delta^9$ ,<sup>11</sup>-cholenic acid.

7. Treatment of this series of compounds with bromine indicates the position of the double bond. Compounds with double bonds  $C_3:C_{11}$  absorb 1 or more moles of bromine which are almost completely removed in a mixture of chloroform and sodium bicarbonate. Compounds with a double bond  $C_{11}:C_{12}$  add 1 mole of bromine to give dibromo derivatives in which the halogen is stable to sodium bicarbonate.

8. Construction of Stuart models shows that the cyclic ether in 3,9-epoxy- $\Delta^{11}$ -cholenic acid can be formed without distortion if the Rings A and B have a cis arrangement, as in *cis*-decalin, and if the hydroxyl group at  $C_3$  is  $\alpha$ ; that is, on the side of the molecule opposite the methyl group at  $C_{10}$ . Formation of the 3,9-epoxy compound is good confirmation for the configuration previously assigned for the structure of the bile acids.

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# A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS\*

## VIII. THE ISOLATION OF THE ANTISTIFFNESS FACTOR FROM CANE JUICE

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The existence of a fat-soluble dietary factor essential for guinea pig nutrition was well established through investigations by Wulzen and Bahrs (1-3) and has been confirmed by Anderson and Caldwell.<sup>1</sup> The isolation of a highly active fraction from raw cream, capable of alleviating an induced stiffness in guinea pigs, was described by van Wagtendonk and Wulzen (4). It seems apparent from physiological studies that the factor has a regulatory effect on the phosphorus metabolism. One of the most prominent changes found was a sharp decrease in the easily hydrolyzable phosphorus fraction in the liver and kidneys during the deficiency. This fraction responded immediately to the administration of the antistiffness factor to deficient animals in that the values returned to normal after a short time of treatment (5). Similar changes were observed in the concentration of the acid-soluble phosphorus in the muscle. The concentrations of creatine phosphate and adenosine tri- and diphosphate are lower in the deficient animal (6). As a result of the deranged phosphorus metabolism other, probably secondary, changes are (1) an increase in the concentration of inorganic phosphorus and calcium in the blood, (2) an increase in calcium in the body tissues (7), and (3) an abnormal distribution of the protein nitrogen in the blood (8).

Only 3 mg. of an oil, curative in a 0.1  $\gamma$  dosage, were obtained from 55 gallons of raw cream (4). Since much larger amounts of raw cream would have been needed for a successful isolation of the antistiffness factor, it was decided to test other possible sources of raw material for the presence of this factor. It was found that crude cane molasses and crude unheated cane juice were good sources of the factor, the latter being around 100 times as active. Due to the unavailability of crude cane juice, cane molasses was first used for the extraction of the factor. Later cane juice became available and was used exclusively.

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<sup>1</sup> Anderson, C. A., and Caldwell, A. L., in press.

We were able to extract and purify a crystalline compound which in a daily dose of 0.002  $\gamma$  would relieve the stiffness induced by the skim milk diet in 5 days. The low level of the easily hydrolyzable phosphorus returned to normal values in the same time.

## EXPERIMENTAL

### *Method of Assay*

Guinea pigs weighing from 350 to 400 gm. were raised on the diet, as described by van Wagtenonk (5). The diet consisted of skim milk powder and water, to which the necessary known vitamins and small amounts of copper and iron had been added. The syndrome developed gradually over a period of some months. Animals in which the syndrome was well developed were used for the test. The usual method of assay consisted of a manual determination of the wrist stiffness in the following way.

The fore leg of the guinea pig on the opposite side from the experimenter was extended posteriorly, close to the body wall of the animal, by pressing the thumb on the olecranon process and at the same time supporting the proximal and distal portions of the leg with the fingers. The leg should be as straight as possible. The disengaged hand of the operator was then used to superextend the foot gently by pressing upward on its medial aspect. The foot of a normal animal would bend easily until it formed a right angle with the leg. The nutritionally deficient animals were very sensitive towards the treatment and manifested pain at once when the foot was forced beyond the point of easy bending. This stiffness disappeared if active fractions were administered to the animals. The results are recorded in terms of a series of arbitrary figures. A normal joint is designated as 4, a completely rigid joint as 1. Intermediate conditions are indicated by such symbols as 1.5, 2, 3, 3+, and 4-. 4P indicates that, although normal mobility has been regained, the joint is still painful under manipulation. The fractions were dissolved in cottonseed oil. In order to express activities in a quantitative way we arbitrarily defined 1 unit as follows: A solution of an active fraction in cottonseed oil contains 1 unit per cc. if, when 1 cc. is administered daily for 5 consecutive days to a sick animal, it cures the affected animal in this time, the stiffness being determined as described above.

The determination of the easily hydrolyzable phosphorus in the liver was also used as an assay. Guinea pigs were anesthetized by intraperitoneal injection of nembutal, and the liver was quickly removed and dropped into a mixture of dry ice and ether. The frozen organ was weighed and ground in a Waring blender in a measured volume of ice-cold 5 per



cent trichloroacetic acid, with 10 cc. of acid for 1 gm. of tissue. The suspension was filtered and the inorganic P in the filtrate determined according to the method of Fiske and Subbarow (9). The easily hydrolyzable P was determined by heating an aliquot for 15 minutes in a boiling water bath with N sulfuric acid, the liberated P being determined as described above. The obtained values were compared with those from the livers of deficient animals of the same age group. An increase of the easily hydrolyzable P in the livers of animals receiving the antistiffness factor was considered an indication of the activity.

TABLE I  
*Activity of Other Materials*

	Activity	Maximum dose tested		Activity	Maximum dose tested
	units per gm.	gm.		units per gm.	gm.
Raw cream	1		Sorghum (stalks)	20	
Fresh kale leaves	1		Corn (stalks)	3	
Vacuum-dried kale (60°)	8		Beet molasses	0	3
Raw potato	0.5	1	Soy bean	0	5
Bakers' yeast (Fleischmann)	0	2	Flax seed	10	
			Corn oil	0	5
Beef muscle	0.5	1	Cane molasses	10	
" liver	0.5	1	" juice	100-1000	
Alfalfa	0.5	1	$\alpha$ -Tocopherol phosphate-disodium salt*	0	0.02
Anaheim vacatone	0	2			
Broccoli	0	2	Wheat germ oil concentrate†	2	

\* Obtained through the courtesy of Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc.

† From Distillation Products, Inc.

*Other Sources of Factor*—Various other raw materials were tested for the presence of the antistiffness factor, according to the first assay method. The results are represented in Table I. The fact that the  $\alpha$ -tocopherol phosphate-disodium salt is inactive in concentrations as high as 20 mg. again definitely establishes the fact that the described deficiency disease is not due to the absence of vitamin E from the diet.

*Isolation from Cane Juice*—The extraction procedure for cane juice was identical with that for molasses. For this reason only the method with cane juice will be given. In total 6 tons of molasses and 7 tons of cane juice were used for the extraction of the antistiffness factor.

*Extraction with Ethyl Ether*—55 gallons of crude cane juice (100 units per gm.; total 20,000,000 units) were extracted at room temperature in a

semicontinuous extraction apparatus.<sup>2</sup> It was found advantageous to pass the cane juice through a stationary column of ethyl ether. In this way, emulsification, although still a serious interference, was considerably less than if the ether were to flow through the cane juice. The extraction tower was filled with wooden blocks ( $2 \times 2 \times 2$  cm., preextracted with ethyl ether) which were thoroughly soaked in cane juice. The tower was then charged with 5 gallons of ethyl ether and the cane juice was passed through it at the rate of 55 gallons per 4 to 5 hours. After six extractions the tower was drained and the emulsion centrifuged to recover the ether extract. The tower was refilled with ether and the extraction continued. A 4 day extraction (four charges of ethyl ether, total twenty-four runs) was sufficient to extract all of the active material. The ether extract of each run was washed three times with 3 liters of water, dried over anhydrous sodium sulfate, and concentrated in a stream of nitrogen. Yield, 17 gm. of a dark green wax; 1,000,000 units per gm.; total 17,000,000 units.

*Distribution between Immiscible Solvents*—The wax obtained from the extraction was dissolved in 1 liter of petroleum ether (Skellysolve H) and four to five times extracted with 1 liter of 90 per cent methanol. The active substance remained in the petroleum ether layer. After drying over anhydrous sodium sulfate the solution was concentrated in a nitrogen atmosphere. Yield, 11 gm. of a green wax; 1,500,000 units per gm.; total 16,500,000 units.

*Selective Adsorption on Magnesium Oxide*—The green wax from the second step was dissolved in 500 cc. of a petroleum ether-benzene mixture (9:1). 25 to 50 gm. of magnesium oxide (adsorptive powdered magnesia, No. 2641, California Chemical Company, Newark, California) were added. The mixture was thoroughly shaken. After centrifuging, the pale yellow supernatant solution containing the active substance was again concentrated in a stream of nitrogen. Yield, 6 gm. of an orange wax; 10,000,000 units per gm.; total 60,000,000 units.<sup>3</sup>

*Molecular Sublimation*—The wax so obtained was submitted to a molecular sublimation in a simple pot still. The condensing surface was cooled with a dry ice-acetone mixture. The vacuum was maintained at 0.1  $\mu$ . Some fractionation was possible. At a bath temperature of 70° a yellow inactive oil condensed at the cooled surface. At a bath temper-

<sup>2</sup> The authors want to express their appreciation to Professor G. W. Gleeson from the Department of Chemical Engineering for his valuable advice in the construction of the extractor unit.

<sup>3</sup> On elution of the magnesium oxide with ethanol, a fraction can be obtained which in very small dosages will increase the degree of stiffness. The removal of this fraction accounts for the increase of the total activity from 16,500,000 to 60,000,000 units in this step.

ature of 140–170° the active substance (mixed with some oil) condensed at the surface of the cold finger. The distillate was dissolved in benzene and 9 volumes of 95 per cent ethanol were added. After standing overnight in the ice box the precipitate was filtered, washed with a cold ethanol-benzene mixture (1:9), and dried in a vacuum desiccator. The major part of the oil was thus removed. The dry material was submitted to a second molecular sublimation. The now white distillate was dissolved in purified petroleum ether<sup>4</sup>. The solution was cooled and the white crystalline precipitate filtered. The material thus obtained usually

TABLE II  
*Stiffness Test*

Dosage	Course of cure													
	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
$\gamma$														
10	2.5	2.5	3	3	4—	4	4	4						
0.01	2.5	2.5	3.5	3—	4—	3+	4	3+	4	4—	4	4P	4	4
0.002	3	2.5	3+	2.5	3.5	3.5	3.5	3.5	3.5	3.5	4—	4—	4	4
Control	2	2.5	2	2.5	2	2.5	2	2.5	2	2.5	2	2.5	2	2.5

TABLE III  
*Easily Hydrolyzable Phosphorus*

Dosage	Easily hydrolyzable P	No. of animals
$\gamma$	mg. per 100 gm.	
10	17.3 $\pm$ 0.5*	10
0.01	16.2 $\pm$ 0.3	10
0.002	8.5 $\pm$ 0.5	5
Control	4.7 $\pm$ 0.2	10

\* The mean values and the standard error are given.

melted from 79–81°. Approximately fourteen recrystallizations from petroleum ether were necessary to obtain a sharp melting product. The purified material melted from 81.5–82°. It consisted of pure white leaflets. Yield, 0.1 gm.; 500,000,000 units per gm.; total 50,000,000 units.

*Activity Tests*—The activity of the crystalline compound was determined as described above. The results are given in Tables II and III.

<sup>4</sup> 2 liters of Skellysolve H were shaken five times with 100 cc. of fuming sulfuric acid, washed acid-free with distilled water, dried over Na<sub>2</sub>SO<sub>4</sub>, anhydrous, and distilled over sodium. The fraction boiling from 62–78° was collected and used for the recrystallizations.

The dosage of 0.002  $\gamma$  can therefore be considered as the minimal dosage necessary to relieve the stiffness and to return the level of the easily hydrolyzable phosphorus to normal values.

#### DISCUSSION

From this and other previously reported investigations (4-7) it becomes apparent that the compound isolated from the cane molasses and the cane juice is able to prevent and to cure the symptoms characteristic for the deficiency. In view of the extremely small dosages required we feel justified in classifying the compound as an essential metabolite. Work towards the elucidation of its structure is in progress. It has not yet been possible to establish whether this compound is also present in the previously isolated fraction from raw cream.

#### SUMMARY

A procedure for the isolation of a crystalline factor present in molasses and raw cane juice, which cures an induced stiffness in guinea pigs, has been described. The abnormal low level of the easily hydrolyzable P in the liver of deficient animals can be returned to a normal value by the administration of minute dosages of the antistiffness factor. The smallest curative dosage is 0.002  $\gamma$ .

The authors want to express their sincere thanks to Dr. Arthur G. Keller of Louisiana State University for his cooperation in obtaining large amounts of cane juice. Thanks are also due to the Pacific Molasses Company, San Francisco, California, for the generous amounts of molasses furnished and to the Aubandon Sugar Manufacturers and the United States Sugar Corporation of Clewiston, Florida, for the cane juice shipped.

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# THE RÔLE OF DIMETHYL- AND MONOMETHYLAMINO-ETHANOL IN TRANSMETHYLATION REACTIONS IN VIVO\*

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An investigation of the metabolic behavior of dimethyl- and monomethylaminoethanol was undertaken as a further study of members of the general class of N-methyl compounds. It was hoped, however, that in the study of these particular compounds some clue might be uncovered as to the mechanism by which choline is synthesized and degraded in the course of participating in transmethylation reactions (1, 2).

In our first exploratory experiments, the growth-promoting properties of both monomethyl- and dimethylaminoethanol were studied. The monomethyl compound proved to be so toxic that its activity could not be adequately tested. In addition to the growth studies on dimethylaminoethanol, data were also obtained on its effect on fatty livers and hemorrhagic kidneys. Finally, the labeling of the methyl groups with deuterium was resorted to in order to gain a closer insight into the fate of the methyl groups of these compounds. After the feeding of the labeled compounds, the tissue choline and tissue creatine were isolated and their deuterium content was determined.

For the preparation of deuteriomonomethylaminoethanol, deuteriomethyl iodide, prepared from deuteriomethyl alcohol (1), was used as the methylating agent of N-*p*-toluenesulfonylaminoethanol. For the preparation of deuteriodimethylaminoethanol, direct methylation with deuteriomethyl iodide could not be employed.<sup>1</sup> Therefore, advantage was taken of the procedure of Clarke, Gillespie, and Weiss Haus (4) for the methylation of amines with formic acid and formaldehyde. Deuterioformic acid, obtained by pyrolysis of deuteriooxalic acid (5), was used with ordinary formaldehyde to methylate aminoethanol. In this way, the methyl group is labeled by the partial replacement of 1 hydrogen atom by deuterium. This synthesis has the advantage of using a readily available source of the

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<sup>1</sup> Frankel and Cornelius (3) have reported the preparation of dimethylaminoethanol, isolated as the gold chloride, subsequent to the treatment of aminoethanol with a large excess of methyl iodide in the presence of sodium hydroxide. In our laboratory, choline was the only product obtained from the reaction mixture.

isotope, since deuteriooxalic acid can be prepared by a simple exchange reaction between deuterium oxide and oxalic acid.

#### EXPERIMENTAL

*Preparation of Deuteriomonomethylaminoethanol* ( $CD_3NHCH_2CH_2OH$ )—Deuteriomethyl iodide, obtained from 6.3 gm. of deuteriomethyl alcohol (1), was distilled directly into a solution of 32 gm. of N-*p*-toluenesulfonylaminoethanol (6) in 100 cc. of 3.5 N sodium hydroxide. The resulting mixture was heated in a well stoppered flask at 65–75° for 90 minutes and then allowed to cool to room temperature. The solution was extracted several times with chloroform, and the chloroform extract was concentrated to dryness *in vacuo*. An alcoholic solution of the residue was decolorized with Darco, and the alcohol was evaporated. 31 gm. of N-*p*-toluenesulfonyldeuteriomethylaminoethanol were obtained.

28 gm. of this compound in 80 cc. of concentrated hydrochloric acid were heated in a sealed tube for 23 hours. The *p*-toluenesulfonic acid which crystallized when the acid solution was cooled to 0° was removed by filtration. The filtrate was concentrated *in vacuo* to remove excess HCl. The deuteriomonomethylaminoethanol hydrochloride was dissolved in absolute alcohol and precipitated with dry ether. 8.0 gm. of product were obtained.

For deuterium analysis, this extremely hygroscopic salt was converted to the picrate. A sample of deuteriomonomethylaminoethanol hydrochloride was dissolved in concentrated potassium hydroxide solution and the free base was extracted with ether from the alkaline solution. The ether extract was dried over sodium sulfate and was treated repeatedly with an ether solution of picric acid. The picrate which precipitated was extracted with boiling ether to remove excess picric acid.

*Analyses*—Deuteriomonomethylaminoethanol picrate

Elementary.<sup>2</sup> Calculated,<sup>3</sup> C 35.11; found, C 35.56

Deuterium. 23.8 ± 0.5 atom% (95.2 atom% in methyl group)

*Preparation of Deuteriodimethylaminoethanol* ( $((CH_2D)_2NCH_2CH_2OH)$ )—Aminoethanol was methylated by the method of Clarke and coworkers (4) for the methylation of amines. Deuterium was introduced by the use of deuterioformic acid (DCOOD) in place of ordinary formic acid.

<sup>2</sup> In the combustion of deuterium-containing compounds by the usual microanalytical techniques, deuterium is retained in the combustion tube. Therefore, the hydrogen values of such compounds obtained in the usual manner are unreliable. For this reason, only the values for carbon have been determined for the test compounds.

<sup>3</sup> All calculated values are based on the increased molecular weight due to deuterium in the molecule.

55 gm. of anhydrous oxalic acid were equilibrated with 25 gm. of 99.6 per cent  $D_2O$ . To this mixture were added 2 cc. of glycerol, and the deuteriooxalic acid was converted to deuterioformic acid by heating the mixture in an oil bath at  $180^\circ$ . 40 cc. of solution containing 22 gm. of deuterioformic acid were obtained by distillation and were subsequently treated with 13 gm. of trioxymethylene. The resulting solution was cooled in an ice bath and 12 gm. of aminoethanol were added. The temperature was allowed to rise slowly to avoid violent reaction, and finally the mixture was heated under a reflux for 4 hours. A slight excess of the calculated amount of anhydrous HCl was added to the solution and the volatile material was removed by distillation. The exchangeable deuterium in the residue was removed by repeated equilibrations with ordinary water. An aqueous solution of the final residue was then made alkaline and the mixture was distilled. The distillate was saturated with potassium hydroxide and the dimethylaminoethanol layer was removed. Even after drying over anhydrous potassium carbonate, the deuteriodimethylaminoethanol still contained some water which could not be readily removed by fractional distillation. The product was found to contain 84 per cent dimethylaminoethanol by titration with standard acid and by determination of its nitrogen content.

For deuterium analysis, the dimethylaminoethanol was converted to the picrate.

*Analyses*—Deuteriodimethylaminoethanol picrate

Elementary.<sup>2</sup> Calculated,<sup>3</sup> C 37.62; found, C 37.80

Deuterium.  $5.35 \pm 0.07$  atom % (12.5 atom % in methyl group)

The introduction of deuterium in the preparation of dimethylaminoethanol by this procedure was attempted with ordinary formic acid in a medium of  $D_2O$ . The dimethylaminoethanol obtained by this procedure contained only a trace of deuterium.

### *Exploratory Feeding Experiments*

*Growth Experiments with Dimethylaminoethanol*—Growth tests with dimethylaminoethanol were carried out with young rats on methionine- and choline-free diets. The diet was the amino acid diet employed by Chandler and du Vigneaud (7) and contained homocystine (1.25 per cent) as the only sulfur amino acid. The fat-soluble vitamins were mixed in the diets in the amounts used by Chandler and du Vigneaud (7), and the water-soluble vitamins were given as a supplement in pills. The rats received two pills daily; each pill contained 0.01 mg. each of thiamine chloride, riboflavin, nicotinic acid, 0.005 mg. of pyridoxine hydrochloride, 12.5 mg. of choline-free ryzamin-B (8), and 150 mg. of dextrin. The

daily food consumption, the initial body weight, and the average daily change in body weight for each rat are given in Table I.

*Effect of Dimethylaminoethanol on Fatty Degeneration of Liver*—The fat content of the livers of the rats fed dimethylaminoethanol was determined by the method of Best, Channon, and Ridout (9). The results of these analyses are given in the last column of Table I.

TABLE I  
*Growth and Lipotropic Effects of Dimethylaminoethanol*

Rat No. and sex	Days on diet	Daily supplement to basal diet		Average daily food consump- tion	Initial body weight	Average daily change in body weight	Liver fat
			mg.	gm.	gm.	gm.	per cent
233 ♀	24	No supplement		4.6	69	-0.1	27.5
129 ♂	20	Dimethylaminoethanol	50	4.5	71	-0.4	5.7
130 ♀	20	"	50	5.3	57	-0.4	5.1
231 ♂	24	"	25*	5.1	77	+0.4	3.8
232 ♂	16	"	25*	5.0	79	+0.4	8.3
232 ♂	8	"	50*	4.0	85	+0.4	
255 ♂	20	"	50	5.4	67	+0.1	4.7
256 ♀	20	"	50	5.0	68	0.0	6.4
327 ♀	8	Choline chloride	50	4.2	40	+1.2	6.7
328 ♀	8	" "	50	4.7	36	+1.0	7.2
268 ♀	16	" "	50	3.6	36	+1.2	

\* Compound injected subcutaneously.

TABLE II  
*Effect of Dimethylaminoethanol on Development of Hemorrhagic Kidneys*

Dietary supplement (10 rats in each group)	Average body weight			Average daily food intake	Average kidney weight	Kidney lesions
	Initial	5th day	8th day			
	gm.	gm.	gm.	gm.	gm.	
None.....	37.3	51.0	49.6	4.2	0.89	7 (Severe) 3 (Partial)
Dimethylaminoethanol....	38.1	47.5	51.3	4.3	0.59	0

*Effect of Dimethylaminoethanol on Hemorrhagic Degeneration of Kidneys*—Two groups of ten rats each were fed the 18 per cent casein diet described by Griffith and Mulford (10) and called by them Diet AC-39. As used in our experiment, the Griffith and Mulford diet was modified as follows: fortified fish liver oil was replaced by corn oil solutions of vitamins A, D, E, and K in the amounts described previously (11), and the water-soluble vitamin supplement also was altered so that each rat received daily an aqueous solution containing 0.02 mg. each of thiamine chloride, riboflavin,



nicotinic acid, and pyridoxine hydrochloride, and 0.2 mg. of *dl*-calcium pantothenate.

One group of rats was kept on this regimen as a control group, while the other group was given, in addition, 48 mg. of dimethylaminoethanol per rat per day. After 8 days, the animals were sacrificed and autopsies were performed. The change in body weight, the average daily food consumption, and the incidence of renal lesions in each group are given in Table II.

### *Feeding Experiments with Labeled Compounds*

In each experiment one male rat and one female rat were fed a synthetic, methyl-free diet of the following percentage composition: amino acid mixture (8) 20,<sup>4</sup> dextrin 27, sucrose 15, salt mixture (Osborne and Mendel (12)) 4, agar 2, corn oil (Mazola) 30. The remaining 2 per cent of the diet varied in each experiment. Vitamins A, D, E, and K were included in the corn oil, as described previously (11). The water-soluble vitamins were administered in aqueous solution. Each rat was fed by stomach tube 0.4 cc. of solution daily, containing 0.02 mg. each of thiamine chloride, riboflavin, nicotinic acid, and pyridoxine hydrochloride, 0.2 mg. of *dl*-calcium pantothenate, and 25 mg. of choline-free ryzamin-B (8). The test compound dissolved in the solution of the B complex vitamins was given to each animal during the experimental period in an amount equivalent in methyl groups to 50 mg. of choline chloride per day. At the end of the experimental period, the rats were sacrificed and choline and creatine were isolated (1) from the tissues of each animal, and the deuterium content of the isolated compounds was determined. The purity of the isolated choline chloroplatinates was determined by platinum analyses, while that of the isolated creatinine potassium picrates was determined colorimetrically by the Jaffe reaction.

*Feeding of Deuteriodimethylaminoethanol with Homocystine*—The diet fed the two rats had the following percentage composition: basal methyl-free diet 98, *dl*-homocystine 0.6, *l*-cystine 0.4, and dextrin 1.0. For 1 week prior to the feeding of deuteriodimethylaminoethanol, the animals each received 50 mg. per day of choline chloride with the B complex vitamins. Then choline was omitted from the aqueous vitamin solution and deuteriodimethylaminoethanol (48 mg. per day) was substituted; enough hydrochloric acid was added to the solution to neutralize it. Table III gives the change in body weight of each rat during the 21 day experi-

<sup>4</sup> The apparent percentage of amino acid mixture in this diet differs from that previously described (7) because the present diet contains 1.3 per cent of *l*-leucine instead of 2.6 per cent *dl*-leucine, 3.8 per cent of *dl*-lysine hydrochloride instead of 4.5 per cent *dl*-lysine dihydrochloride, and 2.3 per cent of sodium bicarbonate instead of 3.9 per cent.

mental period and the deuteriomethyl content of the isolated choline and creatine.

*Analyses*—Choline chloroplatinate

Elementary. Rat 1105. Calculated,<sup>3</sup> Pt 31.6; found, Pt 30.9

" 1106. " " 31.6; " " 31.3

Deuterium. " 1105.  $3.81 \pm 0.07$  atom %

" 1106.  $3.18 \pm 0.08$  " %

Creatinine potassium picrate

Deuterium. Rat 1105.  $0.31 \pm 0.05$  atom %

" 1106.  $0.24 \pm 0.05$  " %

*Feeding of Deuteriodimethylaminoethanol with Methionine*—In this experiment the diet had the following percentage composition: basal methyl-free diet 98, *dl*-methionine 1.4, and dextrin 0.6. The two rats were fed this diet for 1 week without deuteriodimethylaminoethanol in the aqueous vitamin supplement and then for 3 weeks with this compound. The data obtained concerning the change in body weight of the rats and the deuterium content of the isolated creatine and choline are summarized in Table III.

*Analyses*—Choline chloroplatinate

Elementary. Rat 1103. Calculated,<sup>3</sup> Pt 31.6; found, Pt 31.2

" 1104. " " 31.6; " " 32.1

Deuterium. " 1103.  $2.02 \pm 0.07$  atom %

" 1104.  $1.93 \pm 0.06$  " %

Creatinine potassium picrate

Deuterium. Rat 1103.  $0.04 \pm 0.04$  atom %

" 1104.  $0.07 \pm 0.04$  " %

*Feeding of Deuteriomonomethylaminoethanol*—Two rats were placed on a diet having the following percentage composition: methyl-free basal diet 98, *dl*-homocystine 1.25, and dextrin 0.75. For 1 week the rats each received 50 mg. per day of choline chloride with the B complex vitamins. Then the administration of choline was discontinued and deuteriomonomethylaminoethanol hydrochloride (120 mg. per day), neutralized with sodium carbonate, was substituted in the aqueous vitamin solution.

The rats lost weight on this diet, and on the 12th day Rat 1201 died. At autopsy the kidneys were found to be enlarged, soft, and pale in color, but no other gross pathology was observed. The surviving rat was given a 10 mg. dose of choline chloride in an attempt to prolong life, but it died 5 days later. At autopsy the liver appeared normal, the kidneys were large and pale, the pancreas was very white, the gastrointestinal tract contained much gas and fluid, and the peritoneal cavity also contained considerable fluid. Choline and creatine were isolated from the tissues of both of these animals. The results of this experiment are summarized in Table III.

TABLE III  
Feeding of Deuteriodimethylaminoethanol and Deuteriomonomethylaminoethanol

Rat No. and sex	Compound fed*	Sulfur amino acid in diet	Dura- tion of experi- ment	Change in body weight	Body choline		Body creatine	
					Deuterium in methyl groups (B)	Per cent methyl derived from compound fed (B/A × 100)	Deuterium in methyl groups (C)	Per cent methyl derived from compound fed (C/A × 100)
					atom per cent		atom per cent	
500 ♂	Choline	Homocystine, 1.25%	23	90-100	51.8 ± 0.5	61.3 ± 0.6	20.1 ± 0.5	23.8 ± 0.6
1105 ♂	Dimethylaminoethanol	" 0.6%	21	61-74	5.93 ± 0.11	47.4 ± 0.9	1.21 ± 0.20	9.9 ± 1.6
1106 ♀	"	+ Cystine, 0.4%	21	55-68	4.95 ± 0.12	39.6 ± 1.0	0.91 ± 0.16	7.5 ± 1.3
1103 ♂	"	Methionine, 1.4%	21	50-64	3.14 ± 0.11	25.1 ± 0.9	Trace	
1104 ♀	"		21	55-71	3.01 ± 0.09	24.1 ± 0.7	"	
1200 ♂	Monomethylaminoethanol	Homocystine, 1.25%	17†	75-92	10.72 ± 0.11	11.3 ± 0.1	"	
1201 ♀	"		12‡	73-51	12.65 ± 0.20	13.3 ± 0.2	"	

\* All the compounds were fed at levels equivalent in methyl groups to 50 mg. of choline chloride per rat per day.

† Rat given 10 mg. of ordinary choline chloride on 12th day; died on 17th day.

‡ Rat died on 12th day.

*Analyses*—Choline chloroplatinate

Elementary.	Rat 1200.	Calculated, <sup>1</sup> Pt 31.6; found, Pt 32.1
	" 1201.	" " 31.6; " " 32.2
Deuterium.	" 1200.	6.89 $\pm$ 0.07 atom %
	" 1201.	8.13 $\pm$ 0.13 " %

## Creatinine potassium picrate

Deuterium.	Rat 1200.	0.16 $\pm$ 0.11 atom %
	" 1201.	0.09 $\pm$ 0.06 " %

## DISCUSSION

Although the dimethylaminoethanol exhibited both lipotropic and anti-hemorrhagic activities, the growth experiments demonstrated that dimethylaminoethanol does not supply methyl groups for the synthesis of methionine from homocystine at a rate sufficiently rapid to permit as good growth of the young rat as is obtained when choline is fed. However, under these conditions experiments with the deuteriodimethylaminoethanol demonstrated that the methyl groups of the dimethyl compound are used very efficiently by the rat in the synthesis of choline. About 43 per cent of the methyl groups in the tissue choline were derived from the deuteriodimethylaminoethanol in 3 weeks, when the latter was fed as a supplement to a diet containing homocystine and cystine as the only sulfur-containing amino acids. This figure may be compared with the value of about 60 per cent obtained in experiments in which deuteriocholine was fed with homocystine (2).

The seemingly anomalous situation that the dimethyl compound prevents fatty livers and hemorrhagic kidneys and is a relatively good source of the methyl groups of choline, yet is not an efficient substitute for choline in supporting the growth of young rats on the homocystine diet, would become understandable if the animal were incapable of demethylating dimethylaminoethanol to yield methyl groups for the synthesis of methionine but were capable of methylating it to yield choline. If this should be the case, dimethylaminoethanol would not enter directly into transmethylation reactions, such as the formation of methionine and creatine, yet would be convertible to choline. This concept would carry with it the implication that choline, in engaging in transmethylation, releases only one methyl group, and dimethylaminoethanol is formed. *Dimethylaminoethanol would thus assume a pivotal position as both the immediate precursor and the principal demethylation product of choline.*

In previous experiments it was found that when deuteriomethionine was the only methyl donor in the diet of young rats, the creatine and choline isolated from the rat tissues had about the same deuteriomethyl content (1). If the metabolic pathway of dimethylaminoethanol were such that its methyl groups were only given up to homocystine for the

formation of methionine and subsequently appeared in choline and creatine, this same relationship between the deuterio methyl content of tissue choline and tissue creatine should result when the deuteriodimethylaminoethanol is fed with homocystine. However, the data listed in Table III show that the choline contained much more deuteriomethyl than the creatine. In fact, the distribution of the deuteriomethyl groups between choline and creatine was similar to that obtained in previous experiments in which deuteriocholine was fed (2). The results are more in accord with the hypothesis that dimethylaminoethanol is a precursor of choline but cannot itself be demethylated to yield directly utilizable methyl groups.

On the basis of these concepts, the conversion of the administered dimethylaminoethanol to choline would be accomplished on the otherwise methyl-free diet only at the expense of tissue methionine or some other source of labile methyl groups. Nevertheless, with two of the methyl groups necessary for choline synthesis already incorporated in the diet in the form of dimethylaminoethanol, the dietary situation would undoubtedly be much improved over a completely methyl-free diet. The lipotropic and antihemorrhagic properties and the very limited growth-promoting power of dimethylaminoethanol on the homocystine diet would thus be accounted for. On the other hand, its activity in preventing fatty livers and hemorrhagic kidneys could, of course, reside in the intrinsic activity of the compound itself. Other compounds which have no labile methyl groups are known to exhibit these activities (13). One must, in addition, bear in mind the possibility that intestinal flora might play a rôle in some manner in the utilization of dimethylaminoethanol.

The direct conversion of dimethylaminoethanol to choline has been suggested by Jukes and Oleson (14, 15) to explain their experiments on the growth-promoting and antiperotic activities of dimethylaminoethanol in chicks. These investigators suggest that dimethylaminoethanol may exist in some normal diets of chicks and in other natural sources.

It is of interest to note that the appearance of the labeled methyl groups in the choline of the tissues occurred after the feeding of deuteriodimethylaminoethanol, even when the homocystine and cystine in the diet were replaced by methionine. Thus, in spite of the availability of a more than adequate supply of labile methyl groups in the form of methionine, 23 per cent of the methyl groups of choline was still derived from dimethylaminoethanol, as compared to 43 per cent when dimethylaminoethanol was the sole source of methyl groups in the diet (Table III).

The experiments with monomethylaminoethanol were not so extensive as those with the dimethyl compound. Since monomethylaminoethanol was found to be toxic to small rats (about 40 gm.), its effects on growth, fatty liver, and hemorrhagic kidneys were not investigated. In the isotope

experiment (Table III), in which somewhat larger rats were used (about 75 gm.), the animals also lost weight rapidly and died after a short time on a diet in which monomethylaminoethanol was the only possible source of methyl groups. Nevertheless, the choline isolated from the tissues of these animals contained a significant amount of deuterium. The creatine isolated from these rats, however, had a very low deuterium content, consistent with the value obtained when the same isotope level of body choline is attained by feeding deuteriocholine. Again the ratio of deuterium in choline and creatine is the same as after the feeding of choline.

By reasoning analogous to that used for the dimethyl compound, it appears probable that monomethylaminoethanol is also a precursor of choline, but is certainly not itself a direct methyl donor. In the course of the methylation of aminoethanol, shown by Stetten (16) to be a precursor of choline, first monomethyl- and then dimethylaminoethanol may be formed. The hypothesis that aminoethanol is transformed to choline in three successive methylation steps has been suggested by Jukes, Dornbush, and Oleson (17) on the basis of their work with the "*cholineless*" mutant (No. 34486) of *Neurospora crassa*. This work has recently been confirmed and extended by Horowitz (18), who has presented further evidence for the validity of this mechanism of choline synthesis in *Neurospora*.

Stetten (16) has also shown that aminoethanol is not a product of choline degradation. This fact likewise fits in with our concept that the demethylation of choline stops after one methyl group has been transferred to a methyl acceptor such as homocysteine. The difference in behavior of the partially methylated aminoethanols and choline, in so far as the ease with which an intact methyl group may be transferred, may be ascribed to the fact that choline is a quaternary nitrogen compound, while the others are trivalent nitrogen compounds.

#### SUMMARY

The relationship of monomethyl- and dimethylaminoethanol to choline and transmethylation reactions has been investigated. Dimethylaminoethanol was found to be active in preventing the formation of fatty livers and hemorrhagic kidneys but was decidedly inferior to choline in growth-promoting properties on a homocystine diet. Nevertheless, feeding experiments in which the monomethyl- and dimethylaminoethanols were labeled with deuterium in the methyl group proved that these compounds are excellent precursors for the synthesis of choline in the animal body. An explanation has been suggested for the apparently contradictory behavior of dimethylaminoethanol, that is, its efficiency as a precursor of choline and its relative inefficiency in substituting for choline to give growth on methyl-free diets.

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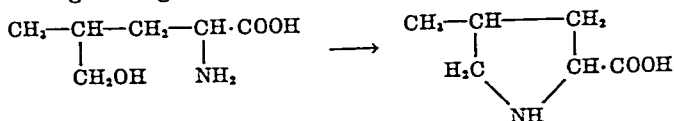
# $\gamma$ -METHYLPROLINE

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The object of the experiments in the following paper was the preparation of  $\delta$ -hydroxyleucine. In a previous communication (1) reference was made to an amino acid derived from hydrolyzed casein which it was thought might possibly be a hydroxyleucine and which was shown definitely to be neither  $\beta$ - nor  $\gamma$ -hydroxyleucine. Unfortunately the synthesis of  $\delta$ -hydroxyleucine has not been accomplished, for, under conditions which might have led to its formation, the product was found to have undergone ring formation with production of  $\gamma$ -methylproline.



This reaction is analogous to Sørensen's observation of the formation of proline from  $\delta$ -hydroxy- $\alpha$ -amino-*n*-valeric acid. It seemed worth while to record a description of  $\gamma$ -methylproline and to state that the evidence for the existence of hydroxyleucine as a product of casein hydrolysis must be regarded as unsubstantial, especially as efforts to convert the amino acid into methylproline have proved unsuccessful.

The condensation of the sodium derivative of acetylaminomalonic ethyl ester<sup>1</sup> with 1,3-dichloroisobutane<sup>2</sup> gave products which on hydrolysis furnished only poor yields of  $\delta$ -chloroleucine.  $\delta$ -Chloroleucine on treatment with 25 per cent sodium hydroxide at 125–130° gave  $\gamma$ -methylproline and no  $\delta$ -hydroxyleucine could be detected. A variety of less drastic methods of replacing the chlorine by hydroxyl was tried but without success.

In order to increase the reactivity of 1,3-dichloroisobutane, one or both chlorine atoms were replaced by iodine by heating with alcoholic sodium iodide. While the replacement of chlorine by iodine was easily accomplished and the iodo compound reacted much more smoothly with the sodium derivative of acetylaminomalonic ethyl ester, the main end-

<sup>1</sup> I am indebted to Merck and Company, Inc., for the gift of acetylaminomalonic ester.

<sup>2</sup> For supplies of 1,3-dichloroisobutane, I am indebted to The Dow Chemical Company.

product of the reaction was  $\gamma$ -methylproline. Incidentally it was found that the products of the interaction of trimethylene iodide and the sodium derivative of acetylaminomalonic ester on suitable treatment gave about 35 per cent of the theoretical amount of proline. The yield of proline from trimethylene bromide is similar. It is of interest to note that the sodium halide formed in the reaction with chloriodoisobutane is chloride rather than iodide. It appears probable that sodium iodide is first formed and then reacts with the remaining chlorine atom. The use of sodium iodide to catalyze the reaction is a practical procedure. Incidentally the observation was made that glycine which invariably accompanies the prolines in the products of hydrolysis gives a relatively sparing reineckate which might easily lead to confusion in the use of Reinecke salt.

#### EXPERIMENTAL

*$\delta$ -Chloroleucine*—After a number of experiments, the following conditions were chosen, although the results left much to be desired. Sodium (4.6 gm.) is dissolved in anhydrous alcohol (75 cc. redistilled over calcium metal). While still slightly warm acetylaminomalonic ethyl ester (43.4 gm.) is added to the solution which is shaken gently until solution is complete. The mixture is then cooled to room temperature or below and with vigorous shaking 2 volumes (150 cc.) of dry dioxane are added by degrees. The sodium derivative of the malonic ester separates out at once. The bulk of the solvents is then removed by distillation under reduced pressure and a temperature not exceeding 40°. The residue is twice treated with additional dioxane (75 cc.) which is removed by distillation as before, thus completing the removal of alcohol. 1,3-Dichloroisobutane in excess (40 gm.) diluted with 2 volumes of dioxane is then added and the mixture heated under a reflux with stirring at intervals in an oil bath at 130° for about 9 hours or until neutrality is reached. Hydrochloric acid (200 cc.) and water (50 cc.) are then added and hydrolysis of the esters completed by boiling under a reflux for 9 hours, an oil bath being used for heating. The solution is then evaporated as completely as possible under reduced pressure and sodium chloride separated with alcohol and removed by filtration. It is possible to isolate some  $\delta$ -chloroleucine from the alcoholic solution of amino acid hydrochlorides by removing chlorine ions with lead hydroxide followed by silver acetate, then removing heavy metals with hydrogen sulfide, and finally converting the amino acids into copper salts in the usual way. The least soluble fraction of copper salts on recrystallization and decomposition gives a little  $\delta$ -chloroleucine, while large amounts of glycine copper salt remain in the mother liquor. In view of the above facts it seemed desirable to remove as much as possible of the glycine before preparation of the copper salts. The only method that

was found satisfactory was to separate it as glycine ethyl ester hydrochloride and to remove it by filtration. The filtrate was then boiled with water, freed from chlorine quantitatively, and finally converted into copper salt with copper carbonate. The copper salt of  $\delta$ -chloroleucine is sparingly soluble in water and crystallizes in hard aggregates of chalky blue needles. The yield amounts to only 6 to 7 gm. It has normal composition without water of crystallization.

$C_6H_{11}NO_2Cl \cdot Cu\frac{1}{2}$ . Calculated, Cu 16.1,  $NH_2-N$  7.14; found, Cu 16.1,  $NH_2-N$  7.21

On decomposing the copper salt, suspended in hot water, with hydrogen sulfide, the free amino acid, crystallizing in needles, was readily obtained. It is only moderately soluble in cold water, freely soluble in hot water, and insoluble in alcohol. Its melting point varies with the speed of heating from 215–220°, giving a brown melt. On heating more strongly, the vapors give a strong pyrrole reaction.

$C_6H_{11}NO_2Cl$ . Calculated. C 43.5, H 7.25, N 8.46  
Found. " 43.3, " 7.15, " 8.50

In view of the possibility of the conversion of  $\delta$ -chloroleucine into  $\gamma$ -methylproline, it may be recorded that on esterification of  $\delta$ -chloroleucine and subsequent hydrolysis no formation of  $\gamma$ -methylproline could be detected.

The chlorine in  $\delta$ -chloroleucine is remarkably strongly bound. Silver acetate in 10 per cent acetic acid does not react at 100°, while at 120° in a sealed tube reduction occurred. Boiling with barium hydroxide solution liberates only a bare trace of chlorine. Heating with water in a sealed tube at 150° for 3 hours results in considerable darkening and liberation of a little over half of the chlorine. A somewhat better result was obtained as follows.  $\delta$ -Chloroleucine (5 gm.) was heated at 125–130° with 15 cc. of 30 per cent sodium hydroxide in a closed steel tube provided with lead washers. About 90 per cent of the chlorine was liberated. On removing sodium as chloride with alcohol and then removing chlorine with silver acetate in customary fashion, it was found that the recovered amino acid was almost all soluble in alcohol (methylproline), while the small insoluble portion was unchanged  $\delta$ -chloroleucine. No evidence of the formation of  $\delta$ -hydroxy-leucine could be secured.

*1,3-Chloroiodoisobutane*—Sodium iodide reacts readily with either one or both chlorine atoms of 1,3-dichloroisobutane. Sealed tubes containing 12.7 gm. of dichloroisobutane (1 mole), alcohol (5 cc.), and 15 gm. of anhydrous sodium iodide (1 mole) were heated at 125–135° for 12 hours. Sodium chloride (5.9 gm.) was filtered off and washed with ether. The contents of three such tubes gave a residue of about 70 gm. On fractiona-

tion, a portion boiling up to  $120^{\circ}$  under normal pressure was first removed. A second fraction (21.5 gm.) boiling at  $120$ – $170^{\circ}$  under atmospheric pressure was next removed and proved to be a mixture of dichloroisobutane and chloriodoisobutane. The third fraction (30 gm.) distilled close to  $90^{\circ}$  under 10 mm. pressure and proved to be mainly chloriodoisobutane. No attempt was made to obtain a completely pure specimen. A small residue of diiodoisobutane remained in the flask and could only be distilled without decomposition at very low pressure.

*$\gamma$ -Methylproline*—The condensation of 1,3-chloriodoisobutane and the sodium derivative of acetylaminomalonic ester was carried out exactly as previously described for the preparation of  $\delta$ -chloroleucine. After hydrolysis and removal of sodium chloride the bulk of the glycine was separated as ethyl ester hydrochloride. The filtrate was diluted with water and evaporated to a syrup. Chloride was then removed with silver acetate and the excess of silver precipitated as sulfide. For the isolation of methylproline two methods were employed. The first involves the use of Reinecke salt, which precipitates  $\gamma$ -methylproline almost quantitatively, and decomposition of the reineckate with pyridine as described by Bergmann for proline. The second method is possibly preferable and simply consists in evaporating the neutral amino acid to dryness and extracting the methylproline with hot absolute alcohol. The alcoholic solution is evaporated and again treated with absolute alcohol to remove any trace of glycine. The  $\gamma$ -methylproline crystallizes readily on concentrating the alcoholic filtrate and may be recrystallized from alcoholic solution by addition of anhydrous ether. It melts at  $230^{\circ}$  with effervescence.

$C_6H_{11}O_2N$ . Calculated.	C 55.8, H 8.60, N 10.9
Found.	" 55.7, " 8.61, " 10.9

Amino nitrogen (Van Slyke) was completely absent. The copper salt of  $\gamma$ -methylproline is much less soluble in water than that of proline and crystallizes in aggregates of plates and prisms with an indigo violet color and contains no water of crystallization. Methylproline is readily precipitated by phosphotungstic acid.

*$\gamma$ -Methylproline Reineckate*— $\gamma$ -Methylproline (0.13 gm) dissolved in water (5 cc.) was added to a solution of Reinecke salt (0.5 gm.) in 10 cc. of water and acidified to Congo red with 2 equivalents of hydrochloric acid. The theoretical amount of reineckate (0.45 gm.) immediately separated as clumps of pink needles. It darkens above  $150^{\circ}$  and melts at  $158$ – $160^{\circ}$ . It is sparingly soluble in water but very soluble in methyl alcohol and insoluble in acetic acid. The melting point is unchanged on recrystallization from aqueous methyl alcohol.

*$\gamma$ -Methylproline Rhodanilate*— $\gamma$ -Methylproline (0.13 gm.) dissolved in 10 cc. of 0.2 N hydrochloric acid was added to 0.5 gm. of ammonium rhodanilate dissolved in 7 cc. of methyl alcohol. The immediate precipitate was small but on standing at a low temperature 0.47 gm. of fine deep red needles separated out. The theoretical yield is 0.64 gm. and the substance appears to be slightly more soluble than the reineckate. It darkens above 125° and melts at 135–136°.

*Phenyl Isocyanate Derivative of  $\gamma$ -Methylproline*— $\gamma$ -Methylproline (0.2 gm.) was dissolved in 2 cc. of normal sodium hydroxide, cooled, and treated with phenyl isocyanate (0.2 gm.) in the usual way. The filtered solution was acidified and gave almost a quantitative yield of the derivative. It was recrystallized from aqueous methyl alcohol and separates as stout prismatic needles melting at 182–183°. It is very sparingly soluble in water but freely soluble in alcohol.

$C_{11}H_{14}O_4N_2$ . Calculated, C 62.9, H 6.45; found, C 63.3, H 6.80

On boiling with normal hydrochloric acid and concentrating the solution the phenylhydantoin derivative is obtained as silky needles melting at 104–105°, sparingly soluble in water.

*Glycine Reineckate*—In using Reinecke salt for the preparation of  $\gamma$ -methylproline in some of the preceding experiments, it was found that the final product was contaminated with glycine. The suspicion arose that glycine formed a fairly sparingly soluble reineckate. This proved to be the case, although this seems surprising in the light of Kapfhammer and Eck's (2) experiences.

Glycine (1/100 mole = 0.75 gm.) with 2 equivalents of hydrochloric acid was treated with 3.5 gm. of Reinecke salt (theory, 3.32) in a total volume of 50 cc. Crystals separated on cooling and after standing at 0° overnight were filtered off and washed with ice water. The bright red (not pink) needles weighed 93 per cent of the theoretical amount (3.80 gm.). On carrying out a similar experiment but in a volume of 100 cc., the yield was 82 per cent of the theoretical amount, while a volume of 200 cc. gave 50 per cent of theory. The substance gave the theoretical amount of glycine on decomposition with pyridine. It may be recrystallized from a little warm water and darkens above 133° and is completely melted at 158–160°. Its solubility in water at 2° was approximately 13.8 gm. per liter. It is evident that glycine reineckate may easily occur as an impurity in less sparingly soluble reineckates.

#### SUMMARY

The synthesis of  $\delta$ -hydroxyleucine has not been accomplished. A method is described for the preparation of  $\delta$ -chloroleucine which on treat-

ment with alkali is converted into  $\gamma$ -methylproline. Improved syntheses of the latter substance are described. The evidence for the existence of a hydroxyleucine among the products of hydrolysis of casein must be regarded as unsubstantial.

Glycine gives a sparingly soluble reineckate which may easily introduce difficulties in the separation of amino acids with Reinecke salt.

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# INFRA-RED ABSORPTION SPECTRA OF STEROIDS

## II. ESTROGENS\*

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In this paper, the infra-red absorption spectra of a number of crystalline natural estrogens are presented and considered in relation to chemical structure. As a group, the natural estrogens are distinguished chemically from other steroids by the benzenoid structure of Ring A, which gives rise to certain infra-red absorption bands by which their spectra may be differentiated from those of other steroids. A study has also been made of a synthetic estrogen, stilbestrol (4,4'-dihydroxy- $\alpha,\beta$ -diethylstilbene).

### Methods

Absorption spectra were obtained with the Hardy infra-red spectrophotometer (1), with the same techniques that were employed in the study of androgens (2). The steroids were prepared for analysis by deposition as solid films on rock salt plates from pyridine solutions, as previously described.

### EXPERIMENTAL

The absorption spectra of the following compounds<sup>1</sup> are presented:  $\alpha$ -estradiol,  $\alpha$ -estradiol-3-monobenzoate,  $\beta$ -estradiol,  $\beta$ -estradiol-3-monobenzoate (Fig. 1); equilenin, equilin, estrone, estriol (Fig. 2); and stilbestrol (Fig. 3).

### Analysis of Spectra

As in the case of the infra-red absorption spectra of androgens (2), it has been found possible to assign a number of absorption bands in the spectra of estrogens to specific interatomic vibrations. These bands will be discussed first, after which certain other bands will be considered in relation to the structural configurations characterizing the compounds under study.

*O-H Absorption*—Since all the compounds studied have one or more hydroxyl groups, all their spectra have absorption bands in the region near

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<sup>1</sup> We are indebted to Dr. Erwin Schwenk of the Schering Corporation for the compounds included in this study.

3  $\mu$ , attributable to the linear vibration of O—H bonds. The wavelengths of the absorption maxima observed in this region are listed in

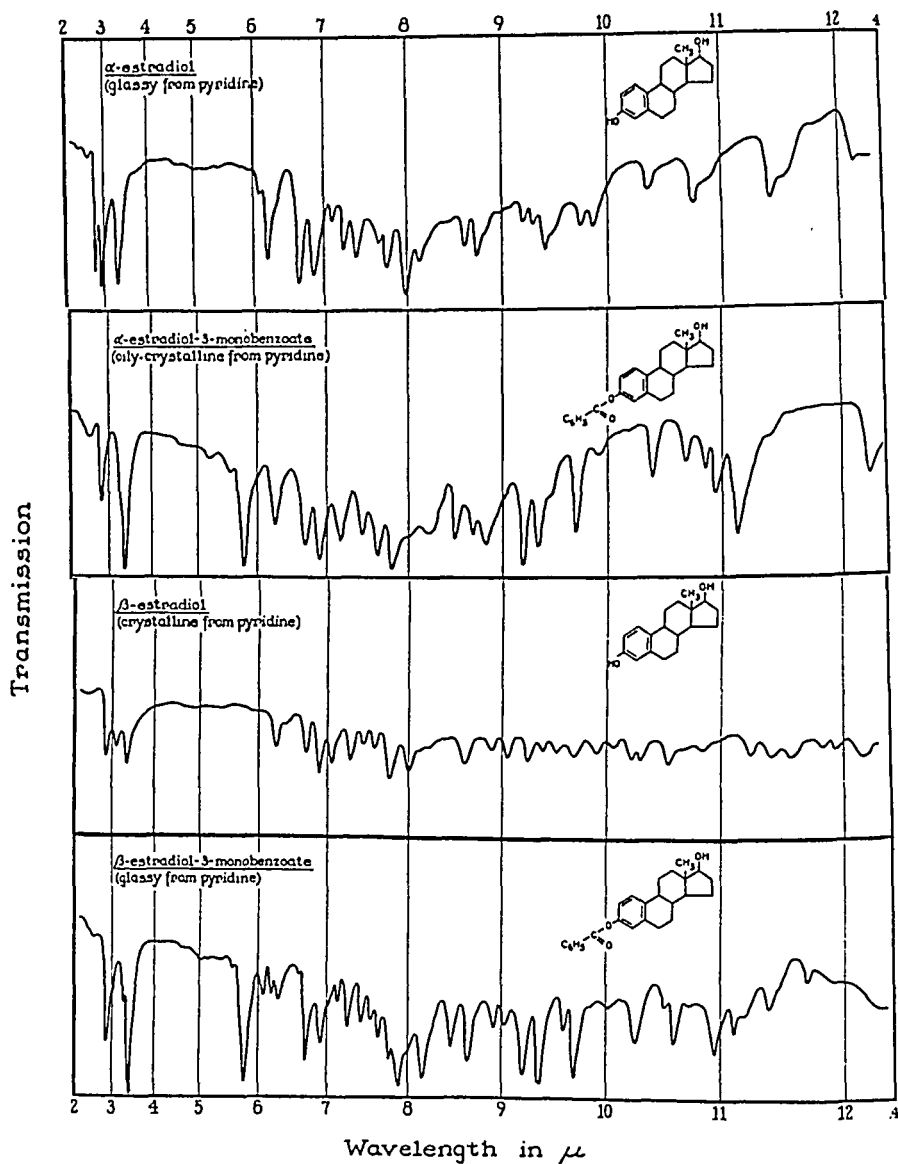


FIG. 1

Table I. All of the wave-lengths, except that for stilbestrol, are significantly higher than the 2.75 to 2.77  $\mu$  absorption range for unbonded O—H



groups (3). As postulated in the case of androgens containing hydroxyl groups (2), this divergence may be the result of hydrogen bonding of O—H

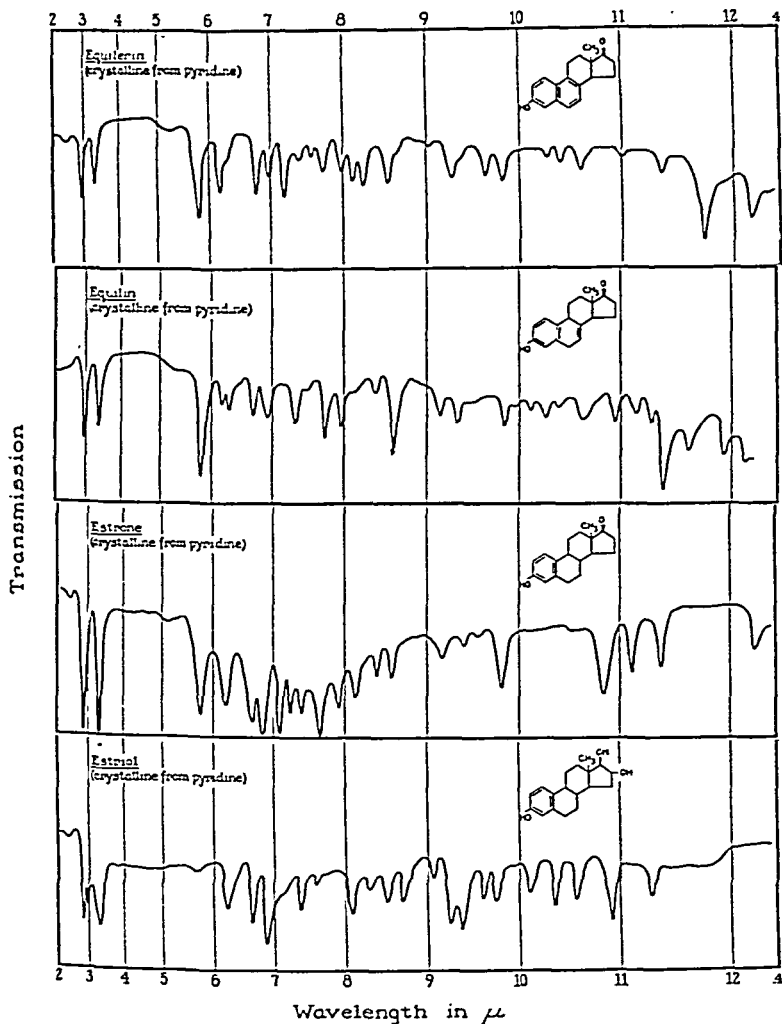


FIG. 2

groups in the solid films of these natural estrogens. The wide range over which the absorption maxima occur may be due to the varying strengths

of hydrogen bonding, since the extent of the shift of O—H absorption varies directly with the strength of hydrogen bonding.

In general, the steroidal estrogens possessing only a phenolic hydroxyl (estrone, equilin, and equilenin) produced an O—H band at a higher wavelength than those containing only an alcoholic hydroxyl ( $\alpha$ -estradiol monobenzoate and  $\beta$ -estradiol monobenzoate). It is therefore likely that in the spectra of  $\alpha$ -estradiol and  $\beta$ -estradiol the absorption bands at 2.80

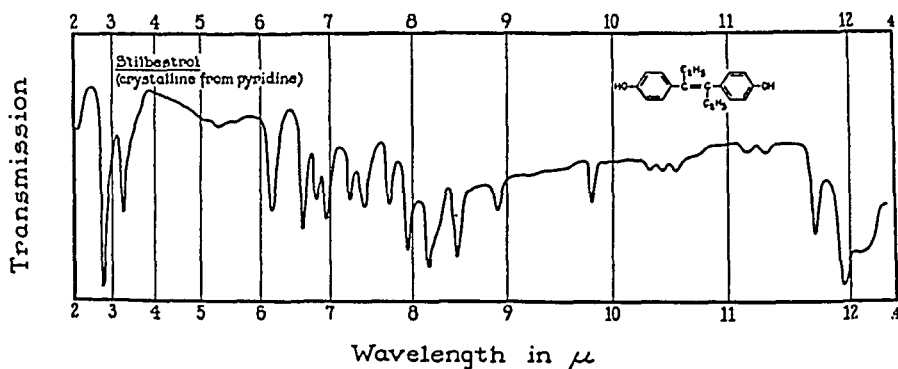


FIG. 3

TABLE I  
*Absorption Maxima in Hydroxyl Region*

Compound	Type of O—H	Wave length $\mu$
Estrone .....	C <sub>3</sub> -Phenolic	2.87
Equilin .....	"	2.95
Equilenin .....	"	2.94
$\alpha$ -Estradiol .....	C <sub>17</sub> -alcoholic	2.80, 2.93
$\beta$ -Estradiol .....	"	2.86, 3.09
Estriol .....	C <sub>17</sub> - and C <sub>16</sub> -alcoholic	2.86, 2.98
$\alpha$ -Estradiol-3-monobenzoate...	C <sub>17</sub> -Alcoholic	2.83
$\beta$ -Estradiol-3-monobenzoate...	"	2.83
Stilbestrol .....	4- and 4'-phenolic	2.78

and 2.86  $\mu$  respectively arise from the alcoholic, whereas those at 2.93 and 3.09  $\mu$  arise from the phenolic hydroxyl groups.

*C—H Absorption*—All the steroids in this series give a major band near 3.35  $\mu$ . This band, as was pointed out in the case of androgens (2), arises from the linear vibration of hydrogen in aliphatic C—H groups. Other workers (4, 5) have shown that C—H groups of benzene rings absorb nearer 3.25  $\mu$  than 3.35  $\mu$ . It might therefore be expected that in the

spectra of estrogens containing benzenoid rings a separate band would appear near  $3.25 \mu$  in addition to the aliphatic C—H band near  $3.35 \mu$ . Such a band, however, does not appear. Hence, it may be concluded that aromatic and aliphatic C—H absorption bands arising from linear hydrogen vibrations have not been resolved in the present study. Likewise in the case of the spectrum of stilbestrol, aromatic and aliphatic C—H bands are not resolved. However, in this compound the aromatic C—H band would appear to predominate, since the absorption maximum occurs around  $3.28 \mu$ .

Absorption bands also arise in the region near  $7 \mu$  from the angular vibration of hydrogen in C—H groups. In this region, the absorption bands resulting from aromatic and aliphatic C—H groups have been clearly resolved. In the spectra of estrone,  $\alpha$ -estradiol,  $\beta$ -estradiol, estriol, and equilin, the aromatic C—H bands occur in the  $6.65$  to  $6.70 \mu$  range. In the case of the monobenzoates of  $\alpha$ -estradiol and  $\beta$ -estradiol, in which the C—H groups of the benzoate structure also contribute to the absorption, the band occurs at  $6.70 \mu$ . The presence of the two double bonds in Ring B of equilenin apparently causes an upward shift of the band to  $6.76 \mu$ , while in the case of stilbestrol, with its para-substituted benzene rings, the band occurs at  $6.62 \mu$ .

The bands which may be assigned to aliphatic C—H angular vibrations occur in the  $6.85$  to  $6.90 \mu$  range in estrone,  $\alpha$ -estradiol,  $\beta$ -estradiol, estriol,  $\alpha$ -estradiol monobenzoate, and  $\beta$ -estradiol monobenzoate. In equilin and equilenin, they are higher, occurring at  $6.93$  and  $6.97 \mu$  respectively. With stilbestrol, as with a number of androgens (2), two aliphatic C—H bands are resolved: one at  $6.81 \mu$  and the other at  $6.95 \mu$ .

**CH<sub>3</sub> Absorption**—In the spectra of androgens and related steroids (2), a band consistently occurred in the region between  $7.20$  and  $7.25 \mu$ . On the basis of the work of Barnes *et al.* on the absorption of methyl group (4), this band was attributed to the angular methyl groups of the androgenic steroids. However, of the estrogens only the following give rise to an absorption band in this specific region: estrone ( $7.23 \mu$ ),  $\alpha$ -estradiol ( $7.23 \mu$ ), and  $\beta$ -estradiol-3-monobenzoate ( $7.23 \mu$ ). In the remainder, the bands closest to this region are, for  $\alpha$ -estradiol-3-monobenzoate,  $7.17 \mu$ ; for  $\beta$ -estradiol,  $7.28 \mu$ ; for estriol,  $7.17 \mu$  (shoulder); for equilin,  $7.32 \mu$ ; and for equilenin,  $7.18 \mu$ . The reasons for this variability are not apparent.

In the spectrum of stilbestrol, there is little doubt that the band of medium intensity at  $7.23 \mu$  arises from absorption by the two methyl groups of the ethyl side chains.

**C=O Absorption**—The only carbonyl groups encountered in the present study are C<sub>17</sub> ketones and ester carbonyls. The former type is present in

estrone, equilin, and equilenin. In studies on androgens and related steroids (2), the  $C_{17}$  ketone gave rise to an absorption band at about  $5.75 \mu$ . The  $C_{17}$  ketone of estrone also absorbs at this wave-length. However, the ketone absorption bands of equilin and equilenin occur at about  $5.80 \mu$ . The increased unsaturation of Ring B of these two compounds may be responsible for this upward shift in the wave-length.

The ester carbonyls of  $\alpha$ -estradiol-3-monobenzoate and  $\beta$ -estradiol-3-monobenzoate both give rise to an absorption band at about  $5.77 \mu$ .

**C=C Absorption**—Aromatic rings are characterized by bands in the region of  $6.20$  to  $6.30 \mu$  (4). Such bands arise from the vibration of conjugated C=C groups in the ring. Since all the compounds under investigation here have one or more aromatic rings, their spectra may be expected to show bands in this region, and such is the case. In most of the spectra a single strong band is found. With equilin and equilenin two bands have been resolved in this region, one of those in equilenin being a small shoulder. The possible origin of these differences is the additional unsaturation of Ring B in these latter compounds.

**C—O Absorption**—In the compounds under investigation, C—O groups may be divided into two classes: (a) those having a carbon with a double bond linkage, and (b) those having a carbon with only single bond linkages. To the first class belong the C—O groups found in phenolic hydroxyl structures. Because of the double bond linkage to the carbon, such groups may be expected to give an absorption band near  $8.0 \mu$  (2, 4). Estrone,  $\alpha$ -estradiol,  $\beta$ -estradiol, estriol, equilin, equilenin, and stilbestrol all contain phenolic hydroxyl groups, and all have an absorption band of medium to strong intensity between  $7.95$  and  $8.05 \mu$ . In the case of  $\alpha$ -estradiol-3-monobenzoate and  $\beta$ -estradiol-3-monobenzoate, the phenolic hydroxyl is esterified. Since each ester linkage has the configuration  $\begin{smallmatrix} \parallel & \parallel \\ \text{C} & \text{—O—C} \end{smallmatrix}$ , each possesses two C—O linkages with double bonds attached to the carbons. These C—O groups probably account for the band of strong intensity at  $7.82 \mu$  in the spectrum of  $\alpha$ -estradiol-3-monobenzoate and at  $7.89 \mu$  in the spectrum of  $\beta$ -estradiol-3-monobenzoate.

The second class of C—O groups (those having a carbon with only single bond linkages) includes C—O groups found in alcoholic hydroxyl structures. They may be expected to give absorption bands in the  $9.0$  to  $10.0 \mu$  region (2, 4). Such groups are present in  $\alpha$ -estradiol, estriol,  $\beta$ -estradiol,  $\alpha$ -estradiol-3-monobenzoate, and  $\beta$ -estradiol-3-monobenzoate.

$\alpha$ -Estradiol which has a  $C_{17}$ -hydroxyl trans to a  $C_{13}$ -methyl, gives a band at  $9.41 \mu$ . This is in accord with the inference made in Paper I (2) that steroids with a  $C_{17}$ -hydroxyl trans to a  $C_{13}$ -methyl give a band in the region  $9.35$  to  $9.45 \mu$ .

In  $\beta$ -estradiol, the alcoholic hydroxyl at  $C_{17}$  is cis to the  $C_{13}$ -methyl. This is the same configuration as that of the  $C_{17}$ -hydroxyl of androstenediol-3( $\beta$ ),17( $\beta$ ):<sup>2</sup> (2). However, in the spectra so far obtained of these two steroids, there is no one outstanding band at a common wave-length among the several small to medium bands in the 9.0 to 10.0  $\mu$  region. Because of this circumstance, a present prediction is unwarranted as to which band in the 9.0 to 10.0  $\mu$  region of the spectrum of  $\beta$ -estradiol results from the C—O linkage of the  $C_{17}$ -hydroxyl.

The spectrum of estriol, which contains a  $C_{17}$ ( $\beta$ )-hydroxyl as well as a  $C_{13}$ -hydroxyl, contains two major bands at 9.27 and 9.37  $\mu$ . These bands probably originate from the hydroxyl groups in question. However, at present, neither band can be assigned to a specific hydroxyl group because of the absence of other reference spectra of steroids containing  $C_{13}$ -hydroxyls and the uncertainties of interpretation discussed in the previous paragraph.

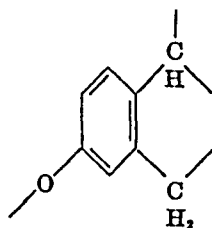
Another type of difficulty is encountered in the assignment, in the 9.0 to 10.0  $\mu$  region, of absorption bands to the  $C_{17}$ -hydroxyls of  $\alpha$ - and  $\beta$ -estradiol-3-monobenzoate. In their spectra, the three strong absorption bands with maxima near 9.20, 9.35, and 9.70  $\mu$  appear to arise from the benzoate portion of the molecule. Their positions and intensities are such as to mask absorption bands of lesser intensity arising from the C—O vibrations of the  $C_{17}$ -hydroxyl groups.

*Other Absorption Bands*—In the spectra of this group of compounds, there are many absorption bands which cannot as yet be assigned to specific interatomic vibrations. However, as in the case of the androgen series (2), certain of these bands can be attributed to unspecified vibrations within specific polyatomic structural configurations of the molecules. This can be arrived at by cross-comparisons of the spectra, and a correlation of common structural configurations and wave-lengths of absorption.

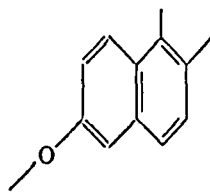
By such cross-comparisons, it was found that a band of medium to strong

<sup>2</sup> In the first paper of this series (2), this compound was incorrectly referred to as  $\Delta^5$ -androstenediol-3( $\alpha$ ),17( $\beta$ ). This error in nomenclature arose from our misinterpretation of the labeling of this sample, which was received from Ciba Pharmaceutical Products, Inc., in 1942 as 3(t),17(c)-androstenediol. It was assumed that the 3(t)-hydroxyl referred to was trans to the  $C_{13}$ -methyl, and that the 17(c)-hydroxyl was cis to the  $C_{13}$ -methyl. The compound was therefore called  $\Delta^5$ -androstenediol-3( $\alpha$ ),17( $\beta$ ), in conformity with the Fieser system of nomenclature. We have since learned that this sample had been labeled under the Ruzicka system of nomenclature, according to which a 3(t)-hydroxyl is equivalent to a 3( $\beta$ )-hydroxyl under the Fieser nomenclature. We are indebted to Dr. A. F. St. André of Ciba Pharmaceutical Products, Inc., for calling our attention to this error in nomenclature. The identity of the original sample as  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\beta$ ) was further established by a comparison of its spectrum with that of a sample of  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\beta$ ) recently furnished us by Dr. St. André.

intensity is present in the range 8.60 to 8.70  $\mu$  in all those compounds containing the structural configuration A. This configuration is present in



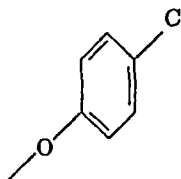
(A)



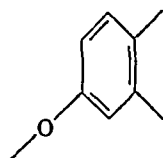
(B)

estrone,  $\alpha$ -estradiol,  $\beta$ -estradiol, estriol, equilin,  $\alpha$ -estradiol-3-monobenzoate, and  $\beta$ -estradiol-3-monobenzoate. In this connection it is of interest that 4-hydroxy-1,2-dimethylbenzene, which has the same configuration, has been reported to give a strong band at about 8.65  $\mu$  (4).

Equilenin, which contains structural configuration B, gives a strong band just below the 8.60 to 8.70  $\mu$  range, at 8.54  $\mu$ . This configuration is the same as is present in  $\beta$ -naphthol, which has been reported to give a strong band at about 8.55  $\mu$  (4). Stilbestrol gives a strong band at 8.48  $\mu$ . Here the structural configuration involved is probably configuration C, since a number of para-substituted phenols and phenolic ethers having the



(C)



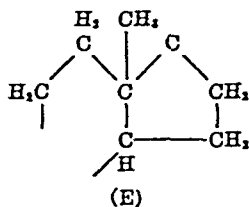
(D)

same configuration give bands in the region 8.45 to 8.52  $\mu$  (4).

In addition to the bands in the 8.60 to 8.70  $\mu$  region, the monobenzoates of  $\alpha$ -estradiol and  $\beta$ -estradiol each give a band in the 8.45 to 8.50  $\mu$  region. It is likely that these bands are attributable to some vibration in the benzoate part of the molecules; however, absorption spectra of other benzoates are necessary to establish this possibility.

A second region in which a band is present in the spectra of all the steroids reported here extends from 11.30 to 11.42  $\mu$ . This band is well defined, though of variable intensity, in all the compounds except  $\alpha$ -estradiol-3-monobenzoate, in which it appears as a shoulder of the strong band preceding it. The bands in the 11.30 to 11.42  $\mu$  region appear to be attributable to some vibration in structural configuration D, which is common to all of the compounds. In assigning these bands to the configuration

D, their possible origin in configuration E, which is also common to all of these compounds, was considered and excluded by cross-comparisons with the spectra of other steroids containing configuration E. Even



though a band occurs in the 11.30 to 11.42  $\mu$  region in some of the spectra of these other steroids, it is not consistently present as in the case with estrogens.

The fact that some steroids other than estrogens give bands in the 11.30 to 11.42  $\mu$  region is not unexpected, since the wave-lengths of absorption arising from different atomic groupings may sometimes be very close to one another. For the same reason, it is also not unexpected that some steroids besides estrogens also give a band in the 8.60 to 8.70  $\mu$  region (see above). This circumstance, however, does not invalidate the usefulness of bands in these two regions in aiding in the identification of estrogens and related steroids. Bands in these two regions, along with a band in the 6.20 to 6.30  $\mu$  region and a band in the 6.65 to 6.70  $\mu$  region, might, together, be considered the "characteristic" bands of these compounds. (Equilenin with bands at 6.21, 6.76, 8.45, and 11.38  $\mu$ , is an exception.) Of all the steroids so far investigated, none, other than those considered in this paper, has a spectrum containing bands in all four of these regions. If an unknown steroid under investigation were to give a band of medium to strong intensity in each of these regions, it would be very strong presumptive evidence that it was an estrogen.

In conclusion, it may be pointed out that all four of these "characteristic" bands of estrogens and related steroids result from vibrations of atoms in the benzenoid ring of the molecules. Thus, the structural configuration which chemically distinguishes this group of steroids from other steroids also distinguishes their spectra from those of other steroids.

#### SUMMARY

1. The infra-red absorption spectra, from 2 to 12.4  $\mu$ , of various estrogens and related steroids have been presented.
2. Certain absorption bands in the spectra have been discussed in relation to the chemical structure of these compounds.

3. Four of these bands, all arising from vibrations of atoms in the benzenoid ring of the molecules, may be used to distinguish the spectra of estrogens and related steroids from those of other steroids.

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# THE ANTISCORBUTIC PROPERTIES OF 3-METHYL-ASCORBIC ACID\*

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During a series of studies of ascorbic acid derivatives an investigation of the physiological activity of 3-methyl-L-ascorbic acid was undertaken. Bezssonov and Sacrez (1) reported in 1937 that this compound showed limited vitamin C activity in the guinea pig. Precise information on this point is of interest since such studies might contribute to a better understanding of the metabolism of ascorbic acid and of the processes involved in making the 3-methyl derivative available to the animal. An "antivitamin" response was also considered as a possibility.

A preliminary report from this laboratory (2) recently pointed out that 3-methylascorbic acid has marked vitamin C activity in the guinea pig. Experimental details and further considerations are presented in this paper.

3-Methyl-L-ascorbic acid was first prepared by Reichstein and coworkers (3) and Haworth and Hirst (4) by the treatment of ascorbic acid with diazomethane. By a similar procedure we have obtained analytically pure, crystalline 3-methylascorbic acid, which has been used in these experiments.

Young male guinea pigs were maintained on a scorbutogenic diet, supplemented daily with 5, 10, or 20 mg. of 3-methylascorbic acid or 2 mg. of ascorbic acid (Merck, U. S. P.). Negative control animals received no supplement or in some cases 50 mg. of glucose per day. In certain groups the supplements were fed in glucose solution by pipette; in other groups the supplements were injected intraperitoneally. Both preventive and curative experiments were carried out. Growth response and blood and adrenal ascorbic acid levels were measured, the latter at the conclusion of experimental periods by the method of Roe and Kuether (5).

In every case graded responses to increasing dosage levels of 3-methylascorbic acid were observed. It is of interest that intraperitoneal administration appeared to be slightly superior to feeding. Daily 20 mg. supplements injected for a period of 75 days led to approximately the same growth responses as were noted by injecting daily 2 mg. supplements of ascorbic

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acid. In spite of such high dosage levels no "antivitamin" effects were noted in this work.

*In vitro* studies were designed with the hope of elucidating the fate of 3-methylascorbic acid in the guinea pig. A series of controlled hydrolyses was carried out under conditions of pH and temperature much more drastic than those existing *in vivo*. No detectable hydrolysis was observed over periods of several hours. The fact that 3-methylascorbic acid exhibits vitamin C activity in the guinea pig is striking in view of this stability. Hydrolysis *in vivo* may, therefore, be accomplished through an enzyme-catalyzed reaction. The high requirement indicates a low efficiency of utilization.

Evidence for hydrolysis is to be found in the presence of ascorbic acid in the blood and adrenal glands of animals in these experiments after 75 days of deficiency. Very high concentrations of the methyl derivative would be required to lead to such apparent levels through interference with the Roe and Kuether method (see "Experimental"). It seems more logical to assume a rapid excretion of 3-methylascorbic acid in the urine, although failure in this work to devise a method for its quantitative determination leaves some doubt at this point.

#### EXPERIMENTAL

*Preparation and Properties of 3-Methyl-l-ascorbic Acid*—l-Ascorbic acid (Merck, U. S. P., 6 gm.) was dissolved in 90 ml. of absolute methanol. The solution was filtered and cooled to approximately  $-40^{\circ}$  by means of a methyl cellosolve- $\text{CO}_2$  bath. An ethereal solution of diazomethane prepared from nitrosomethylurea (6) and similarly cooled was added dropwise from a separatory funnel at a fairly rapid rate until decolorization did not immediately follow each addition. When a precipitate separated near the end of the reaction, the solution was allowed to warm slightly until the material had redissolved before addition of diazomethane was continued. At the end of the reaction the solvent was removed under reduced pressure. Seeding the syrup with a few crystals of 3-methylascorbic acid at this step hastened crystallization materially. After the product had stood for a few hours *in vacuo* to complete crystallization, the solid material was taken up in 130 to 150 ml. of cold acetone. An insoluble residue consisting of unchanged ascorbic acid and 1-methylheteroascorbic acid (7) was removed by filtration. Crystallization was brought about by adding dry low boiling petroleum ether slowly to turbidity. Fine white needles, melting at  $122.0\text{--}122.5^{\circ}$  (corrected) after three recrystallizations, were obtained. The yield of the three times recrystallized product was 64.9 per cent in one preparation.

$\text{C}_7\text{H}_{10}\text{O}_4$  (190.15). Calculated, C 44.21, H 5.30; found, C 44.34, H 5.42

Two 20 mg. samples were titrated with standard alkali with phenolphthalein as an indicator and required 0.89 and 0.88 moles of alkali per mole of compound for neutralization. To account for this reaction the possibility that substitution of a methyl group in the 3 position increases ionization in the 2 position has been suggested by Haworth and Hirst (4).

Heating 3-methylascorbic acid with  $N$  NaOH resulted in the development of a brilliant yellow color which disappeared upon neutralization. It was not possible to employ this color reaction for the quantitative determination of 3-methylascorbic acid in biological material because of the presence of other substances which respond similarly.

*Vitamin C Activity of 3-Methyl-L-ascorbic Acid in the Guinea Pig*—Young male guinea pigs maintained on a scorbutogenic diet of Purina rabbit chow checkers (8) were employed in these studies. Daily supplements of 5, 10, or 20 mg. of 3-methylascorbic acid were administered orally or by intraperitoneal injection. The response was compared with that of negative control animals and of positive control animals receiving 2 mg. of ascorbic acid by either route. Solutions for administration were made up with boiled distilled water immediately prior to use. Oral supplements were given in 2 ml. of a freshly prepared solution containing 50 mg. of glucose.

All preparations of 3-methylascorbic acid were tested for residual ascorbic acid by titrating 40 mg. samples with 2,6-dichlorophenol indophenol. Values within the range, 0.005 to 0.027 mg. of ascorbic acid per 20 mg. of methyl derivative, were found. In view of the ascorbic acid requirement of the guinea pig these amounts are negligible.

Groups of four or more animals were employed to test the various dosage levels and to compare oral with intraperitoneal administration. In addition, both preventive and curative experiments were designed. A preventive experiment involved administration of the test compound daily from the beginning of a period. In curative studies animals were allowed to develop gross symptoms of scurvy before the supplements were added. In the latter tests it is important to begin supplementation before the so called "irreversible" point, beyond which not even ascorbic acid will effect a cure.

The growth response of typical experimental animals is summarized in Figs. 1 and 2. Table I contains a summary of the growth and analytical data for animals on a 3-methylascorbic acid preventive type of experiment, with supplements at the 20 mg. level.

The growth data show conclusively that 3-methylascorbic acid has vitamin C activity in the guinea pig. Curves are shown for twelve out of a total of 68 animals on which complete studies were made. From the growth results it appears that 20 mg. of 3-methylascorbic acid per day make available to the guinea pig slightly less than the equivalent of 2 mg. of ascorbic acid per day.

Observation of the behavior of the animals during experimental periods together with an analysis of the blood and adrenal ascorbic acid levels substantiates the above conclusions. To determine blood and adrenal ascorbic acid the method of Roe and Kuether (5) was employed. The blood analyses were carried out in the usual manner.

The method was adapted for the determination of adrenal ascorbic acid as follows. Upon sacrificing an animal the adrenal glands were carefully removed, weighed quickly, and ground in a mortar with 5 ml. of 1.5 per cent  $\text{CCl}_3\text{COOH}$  and a small amount of washed sand. Filtered extracts and washings were diluted to 25 ml. with 1.5 per cent  $\text{CCl}_3\text{COOH}$ , except

TABLE I

*Effect of 3-Methylascorbic Acid on Blood and Adrenal Ascorbic Acid and Weight Gains*

Guinea pig No.	Supplement*	Method of administration†	Duration of experiment	Adrenal ascorbic acid, total	Blood ascorbic acid	Weight gain, 75 days
			days	mg. $\times 100$	mg. per cent	gm.
63‡			27	0.41	0.05	
71‡			27	0.31	0.03	
73‡			32	0.19	0.03	
39	M.	O.	75	1.50	0.17	290
40	"	"	75	1.94	0.07	320
41	"	"	75	2.00	0.06	323
42	"	"	80	1.75	0.07	230
43	"	I.	76	3.19	0.03	370
44	"	"	76	2.25	0.06	391
45	"	"	82	3.06	0.05	430
46	"	"	80	2.25	0.05	410
47	A.	"	77	9.45	0.17	397
48	"	"	77	9.75	0.16	393
49	"	O.	76	3.88	0.13	422
50	"	"	76	6.43	0.15	383

\* M. = 3-methylascorbic acid (20 mg. per day); A. = ascorbic acid (2 mg. per day).

† O. = oral; M. = intraperitoneal injection.

‡ Negative controls.

in the case of negative control animals in which the dilution volume was 12.5 ml. 10 ml. portions of the diluted extracts were added to 10 ml. of 7.5 per cent  $\text{CCl}_3\text{COOH}$ , and the analyses completed in the usual manner. It was found necessary to prepare the 2,4-dinitrophenylhydrazine reagent fresh each day in order to get consistent results. Table I shows summarized data for three negative control animals, for four positive control animals, and for all the animals receiving 20 mg. supplements of 3-methylascorbic acid in preventive experiments.

The tissue analyses define groups of animals more sharply than do the growth data. In these studies a marked distinction between positive con-

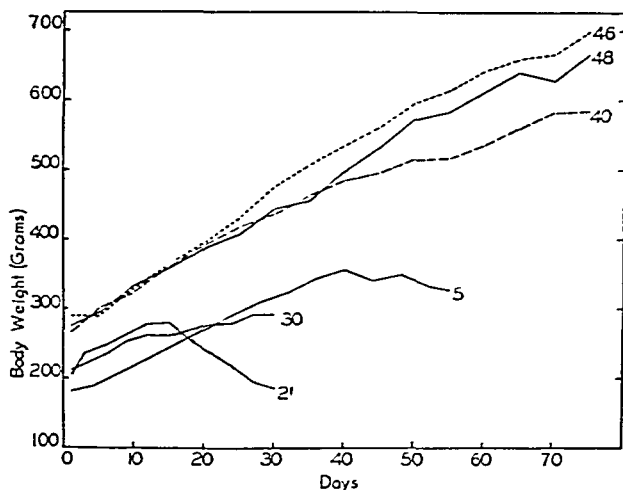


FIG. 1. Preventive experiments. Guinea Pig 5, 10 mg. of 3-methylascorbic acid (oral); Guinea Pig 21, negative control; Guinea Pig 30, 5 mg. of 3-methylascorbic acid (injection); Guinea Pig 40, 20 mg. of 3-methylascorbic acid (oral); Guinea Pig 48, 2 mg. of ascorbic acid (injection); Guinea Pig 46, 20 mg. of 3-methylascorbic acid (injection).

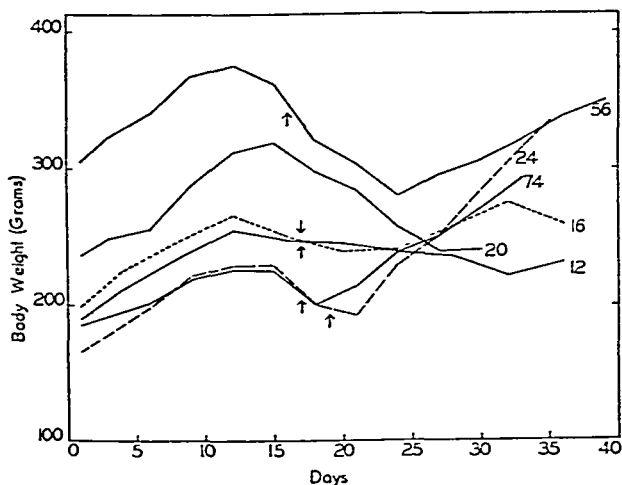


FIG. 2. Curative experiments. Guinea Pig 12, 5 mg. of 3-methylascorbic acid (oral); Guinea Pig 16, 10 mg. of 3-methylascorbic acid (oral); Guinea Pig 20, negative control; Guinea Pig 24, 2 mg. of ascorbic acid (oral); Guinea Pig 56, 20 mg. of 3-methylascorbic acid (oral); Guinea Pig 74, 20 mg. of 3-methylascorbic acid (injection). The arrows indicate the beginning of daily supplementation.

trol animals and the group receiving 20 mg. supplements of 3-methylascorbic acid exists.

The following experiment was designed to test the possibility of interference of 3-methylascorbic acid with the Roe and Kuether method. Varying amounts of analytically pure material were added to samples of fresh rabbit blood. When a concentration of 40 mg. per cent of 3-methylascorbic acid was used, an interference of 0.28 mg. per cent was found. If this interference ratio (143:1) is applied to the adrenal analyses in the case of Guinea Pig 46 (the weight of the adrenal glands = 315 mg.), the presence of 3.2 mg. of 3-methylascorbic acid is indicated. The total amount of ascorbic acid present in normal guinea pig adrenal glands is about 0.05 to 0.10 mg. (see Table I). It seems unlikely, therefore, that the analytical data for adrenal tissue are affected by the presence of 3-methylascorbic acid.

In the preventive experiments of long duration the animals receiving 3-methylascorbic acid were less active than positive controls. This is particularly true of those animals receiving the derivative orally. One member of this latter group showed a definite "scurvy" involvement of the hind limbs, which observation is consistent with the idea that a state of mild vitamin C deficiency existed. In addition, in curative experiments at the 20 mg. level the animals receiving 3-methylascorbic acid seemed to recover more slowly than ascorbic acid controls.

The metabolism of 3-methylascorbic acid is an interesting problem. The high requirement and a consideration of data from the animal experiments which have just been described indicate that the derivative itself is not utilized but undergoes conversion to ascorbic acid in the animal. Such a process appears to be inefficient. Experiments were, therefore, designed to investigate hydrolysis.

*In Vitro Hydrolysis of 3-Methyl-L-ascorbic Acid*—The following procedure was employed in these studies. Samples of 13 mg. of ascorbic acid and 14 mg. of 3-methylascorbic acid were dissolved in 25 ml. portions of conductivity water at a given pH and temperature. Previous to the addition of the above substances the water had been deaerated with wet, oxygen-free nitrogen for 30 minutes. During the experimental period a continuous stream of nitrogen was conducted through all systems and the temperature was controlled by means of a water bath. From time to time 1 ml. samples were withdrawn, acidified, and titrated immediately with standardized 2,6-dichlorophenol indophenol (concentration 50 mg. per 600 ml.).

At pH 1.6 substantially no ascorbic acid was destroyed in the control system. The conclusion that no hydrolysis occurs under these conditions is justified, since no ascorbic acid could be detected in the 3-methyl system.

At pH 11.1 the situation is comparable. However, a slow destruction in the ascorbic acid system was observed so that traces of hydrolysis might have escaped notice.

In 1.0 N NaOH, under which conditions the lactone ring is opened and ascorbic acid destroyed, the results were somewhat irregular, but there were no indications that ascorbic acid was formed from 3-methylascorbic acid. The solution of methyl derivative was observed to turn yellow while that of ascorbic acid itself turned lavender with heating.

*In Vivo Hydrolysis of 3-Methyl-l-ascorbic Acid*—Attempts to demonstrate *in vivo* hydrolysis by a rise in the blood ascorbic acid level of rabbits receiving 10 and 12.2 mg. of the methyl derivative per kilo by way of the ear vein led to negative results. No rise in blood ascorbic acid could be detected when samples were taken at intervals and analyzed by the Roe and Kuether procedure. However, a slow rate of hydrolysis would not be detected under these conditions.

The possibility of an enzyme-catalyzed reaction was considered. Preliminary attempts to isolate an enzyme system from the following guinea pig tissues were made: liver, kidney, adrenal, small intestine, blood. Each material except blood was placed in Ringer-Locke's solution (9), ground briefly with sand, and tested for hydrolytic activity. Control systems contained ascorbic acid. No increase in indophenol-reducing activity was detectable in any system after 3½ hours incubation at 37°. This negative result must, of course, be checked by a more carefully restricted technique.

#### SUMMARY

The antiscorbutic properties of pure, crystalline 3-methylascorbic acid have been investigated in a series of preventive and curative experiments on young male guinea pigs. Daily supplements of 20 mg. of 3-methylascorbic acid, when fed or injected intraperitoneally, provide the guinea pig with slightly less than the equivalent of 2 mg. of ascorbic acid. Intraperitoneal injection results in more efficient utilization of the derivative than oral administration.

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# URINARY EXCRETION OF $\alpha$ -AMINO NITROGEN FOLLOWING INTRAVENOUS ADMINISTRATION OF AMINO ACID MIXTURES

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It has recently been reported (Howe, Unna, Richards, and Seeler (1)) that an amino acid mixture consisting largely of the essential amino acids in their natural form can be administered to dogs intravenously without gross ill effect at much faster rates than can a similar mixture which contains a larger proportion of racemic amino acids. Since the rapid administration of amino acids might result in their increased loss in the urine, as suggested by Elman and Weiner (2), experiments were undertaken to determine whether the practical advantages offered by an increase in the rate of infusion might be offset by a greater loss of amino acids in the urine.

Studies were also carried out on the relation of the urinary excretion of  $\alpha$ -amino nitrogen both to the type of amino acid preparation infused and to the state of protein depletion of the animal. The rate of removal from the circulation was determined by measurement of the plasma amino acid levels, and urines were analyzed for the ten essential amino acids by the procedure of Stokes, Gunness, Dwyer, and Caswell<sup>1</sup> (3).

## Methods

In all experiments on the relation of amino acid excretion to the rate of infusion three healthy adult mongrel dogs weighing 10.3 kilos were used. These animals presumably had normal protein stores, as they had been maintained for several months on a diet of Gaines' dog meal supplemented with horse meat and milk. Food was withheld 18 to 24 hours before each infusion and the interval between infusions was not less than 4 days. The amino acid solutions were injected into a leg vein by means of a variable speed perfusion pump. In one experiment the amino acids were introduced by stomach tube. The dogs were placed in metabolism cages immediately after the injections, and 24 hour urines were collected under toluene. Samples of venous blood were withdrawn immediately before infusion and at intervals of 5, 30, 60, and 180 minutes afterwards.

<sup>1</sup> We are greatly indebted to Dr. J. L. Stokes of the Microbiological Laboratories of Merck and Company, Inc., for all microbiological assays reported in this communication.

The experiments on the relation of amino acid excretion to the state of protein depletion of the dogs were carried out on the same three animals. The dogs were placed on Diet 621, which contains 0.01 per cent nitrogen (Kjeldahl): dextrose 71 per cent, Crisco 21 per cent, Mazola 0.15 per cent, Salt Mixture 1 (U. S. P. XI) 3 per cent, bone ash 2.85 per cent, and cod liver oil 2 per cent. Each dog was given a daily supplement of 1.0 mg. of thiamine, 1.0 mg. of riboflavin, 1.0 mg. of pyridoxine, 20 mg. of nicotinic acid, 40 mg. of calcium pantothenate, 200 mg. of inositol, and 200 mg. of choline chloride. The adequacy of a similar diet, including 30 per cent of casein as the source of nitrogen (Diet 620), for maintenance of dogs has

TABLE I  
*Per Cent Composition of Amino Acid Mixtures*

	Mixture Vuj	Mixture VujN
<i>l</i> (+)-Arginine HCl. . . . .	8.0	8.0
<i>l</i> (+)-Histidine HCl-H <sub>2</sub> O. . . . .	4.0	4.0
<i>l</i> (+)-Isoleucine. . . . .		7.8
<i>dl</i> -Isoleucine. . . . .	10.8	
<i>l</i> (+)-Leucine. . . . .	15.4	17.5
<i>l</i> (+)-Lysine HCl. . . . .	12.3	12.3
<i>l</i> (-)-Methionine. . . . .		5.5
<i>dl</i> -Methionine. . . . .	6.1	1.2
<i>l</i> (-)-Phenylalanine. . . . .		8.7
<i>dl</i> -Phenylalanine. . . . .	6.9	
<i>l</i> (-)-Threonine. . . . .		1.7
<i>dl</i> -Threonine. . . . .	10.8	7.4
<i>dl</i> -Tryptophane. . . . .	1.8	1.8
<i>l</i> (+)-Valine. . . . .		6.1
<i>dl</i> -Valine. . . . .	13.9	
Glycine. . . . .	10.0	10.0
Total. . . . .	100.0	92.0

been reported previously from this laboratory (Silber (4); Seeler and Silber (5)).

The amino acid mixtures were infused after the dogs had been on the protein-free diet for 10, 20, 30, and 35 days. To determine the effect of other dietary protein levels on excretion of intravenously administered amino acids, the dogs were each subsequently fed, in addition to Diet 621, a daily supplement of 10 gm., 20 gm., and finally 60 gm. of casein plus 50 gm. of horse meat for 20 day periods. At the end of each feeding period, an amino acid mixture (VujN) was administered as before, at the rate of 6 mg. of N per kilo per minute.

The composition of the two amino acid preparations is shown in Table I. Mixture Vuj was devised and studied by Madden and Clay (6) and has been used clinically. This mixture contains 50 per cent of its amino acids in racemic form and henceforth will be referred to as the *racemic mixture*. Mixture VujN has been described by Howe *et al.* (1) and is very similar to mixture Vuj in its amino acid pattern, but only 10 per cent of its amino acids is in the racemic form. 8 per cent of the nitrogenous material of this mixture has not been identified, but probably consists of non-essential amino acids in the *l* form. This mixture will be referred to as the *natural mixture*.

The mixtures were administered as 8 per cent solutions at pH 6.8. The nitrogen content of the solutions was approximately 1.1 gm. per 100 cc., of which about 0.84 gm. was  $\alpha$ -amino nitrogen. In all experiments the total amount of nitrogen administered was 220 mg. per kilo. Control urines were collected for analysis once while the dogs were on stock diet and the day previous to each test thereafter.

$\alpha$ -Amino nitrogen determinations were performed by the ninhydrin procedures (Hamilton and Van Slyke (7); Van Slyke, MacFadyen, and Hamilton (8)), total nitrogen by micro-Kjeldahl with selenium and peroxide for digestion, and urea and ammonia nitrogen by the Conway diffusion method (Conway and O'Malley (9)). Serum protein levels were determined by the method of Lowry and Hastings (10).

### Results

The total amounts of  $\alpha$ -amino nitrogen recovered from the urines in 24 hours, following the intravenous administration of the racemic and the natural amino acid mixtures at rates of 2, 6, and 12 mg. of nitrogen per kilo per minute, are shown in Table II. For comparison the 24 hour excretion data following the oral administration of an equivalent amount of the natural mixture are shown. In addition the amounts of  $\alpha$ -amino nitrogen excreted by the dogs during 24 hours of fasting and during 24 hours on a normal diet are given. The figures in parentheses give the percentage recovery in terms of the total amount of  $\alpha$ -amino nitrogen administered.

The difference in  $\alpha$ -amino nitrogen excretion after infusion of the two mixtures at all three rates was striking. The amount of  $\alpha$ -amino nitrogen recovered in the urine was consistently greater after the infusion of the racemic mixture than after infusion of the natural mixture. The amount of  $\alpha$ -amino nitrogen found in the urine after the racemic mixture was given increased in rough proportion to the rate of infusion. When the natural mixture was given intravenously at the slow rate (2 mg. of N per kilo per minute), the amount of  $\alpha$ -amino nitrogen in the urine was no greater than that excreted by the dogs on a normal diet or when the same mixture was

TABLE II

*Urinary Excretion of  $\alpha$ -Amino Nitrogen after Administration of Amino Acid Mixtures, in Mfg. per 24 Hours\**

Dog No.	Fasted	Normal diet	Oral, Mixture VujN	Intravenous, mixture					
				Vuj	VujN	Vuj	VujN	Vuj	VujN
				N, mg. per kilo per min.					
				2	2	6	6	12	12
333	20	85	75 (3)	230 (12)	130 (6)	340 (18)	265 (13)	450 (25)	245 (13)
530	24	85	105 (5)	205 (11)	90 (4)	360 (20)	220 (11)	385 (21)	200 (10)
535	20	30	80 (4)	220 (11)	50 (2)	355 (20)	280 (15)	410 (22)	335 (16)

\* The figures in parentheses represent the per cent of the dose excreted.

TABLE III

*Plasma  $\alpha$ -Amino Nitrogen Levels after Intravenous Infusions (Averages, in Mfg. Per Cent; Two Dogs)*

Diet No.	N	Time after infusion				
		0 min.	5 min.	30 min.	60 min.	180 min.
Mixture VujN						
	mg. per kg. per min.					
Stock	Oral	4.2	5.2	8.5	10.8	5.1
	2	4.8	10.6	7.7	5.6	5.3
	6	5.4	29.0	12.2	8.1	5.4
	12	4.6	38.0	12.8	10.2	6.5
621, 10 days	6	5.1	19.0	11.8	8.4	6.1
20 "	6	5.1	18.8	12.4	8.5	6.0
30 "	6	5.2	23.0	12.3	8.8	6.1
621 + 10 gm. casein per day,	6	5.6	23.5	14.9	10.3	6.1
20 days						
621 + 20 gm. casein per day,	6	4.6	23.2	12.3	8.0	4.8
20 days						
621 + 60 gm. casein and 50 gm. meat per day, 20 days	6	4.8	25.5	12.6	8.2	4.7
Mixture Vuj						
Stock	2	4.1	9.8	6.7	5.5	4.7
	6	6.4	26.6	12.9	9.7	5.9
	12	4.2	36.5	15.0	8.0	5.0
621, 35 days	6	5.7	25.3			6.2

given orally. When the rate of infusion was increased from 2 to 6 mg. of N per kilo per minute the excretion increased about 3-fold. However, in contrast to the results with the racemic mixture, when the rate of infusion of the natural mixture was increased from 6 to 12 mg. of N per kilo per minute, there was no increase in amino acid excretion.

When the control urines and the urines collected after administration of Mixture VujN were hydrolyzed with 20 per cent hydrochloric acid for 6 hours at 120°, the  $\alpha$ -amino nitrogen concentration of the urines increased, but the increase was no greater in the urines of dogs given Mixture VujN than in the control urines. The total amount of "bound"  $\alpha$ -amino nitro-

TABLE IV  
*Weights (Kilos) and Serum Protein Values (Gm. Per Cent) of Dogs on Experimental Diets*

Dog No.	Protein-free Diet 621							
	0 day		10 days		20 days		30 days	
	Weight	Serum protein	Weight	Serum protein	Weight	Serum protein	Weight	Serum protein
333	10.3	5.0	10.0	4.9	9.5	5.2	9.4	4.5
530	10.3	5.0	9.8	5.5	9.0	4.6	8.4	4.9
535	10.3	6.5	9.9	4.9	9.2	5.3	8.9	4.8
	Protein-free Diet 621		Diet 621 + 10 gm. casein per day		Diet 621 + 20 gm. casein per day		Diet 621 + 60 gm. casein and 50 gm. meat per day	
	35 days		20 days		20 days		20 days	
	Weight	Serum protein	Weight	Serum protein	Weight	Serum protein	Weight	Serum protein
333	9.1	4.1	9.15	4.7	9.85	5.1	10.75	5.4
530	8.3	4.3	8.1	4.7	8.8	5.3	10.15	5.55
535	8.5	4.5	8.7	4.8	9.45	5.3	10.75	5.95

gen excreted by the dogs when fed the stock diet averaged 45 mg. per day; when infused at rates of 2, 6, and 12 mg. of N per kilo per minute, 35, 37, and 46 mg. per dog per day, respectively. After Mixture VujN infusion to protein-depleted dogs, 46 mg. of "bound"  $\alpha$ -amino nitrogen were excreted in 24 hours.

5 minutes after the infusions the plasma levels were found to be elevated roughly in proportion to the rate of administration (Table III), but in all experiments the levels were approximately normal at the end of 3 hours. No significant difference in plasma levels was observed after the administration of the two mixtures at the same rates. Following oral administration, the highest plasma concentration was observed after 1 hour.

On completion of the experiments described above, the three dogs were placed on the protein-free diet. The data in Table IV show the effect of this diet on body weight and serum protein levels. Infusion was carried out at 10 day intervals with the natural mixture at a rate of 6 mg. of N per kilo per minute. The amount of  $\alpha$ -amino nitrogen excreted in the urine decreased when the dogs were depleted (Table V). After 20 days on the protein-free diet there was as little amino nitrogen excreted following fairly rapid intravenous administration of the natural amino acid mixture as there was when the mixture was given by mouth to the same dogs before depletion. When the dogs were deprived of protein for 35 days, neither

TABLE V

*Effect of Dietary Protein Level on Excretion of  $\alpha$ -Amino Nitrogen (Mg. Per 24 Hours) Following Intravenous Infusion of Amino Acid Mixtures at 6 Mg. of N Per Kilo Per Minute\**

Dog No.	Protein-free Diet 621						Diet 621 + 10 gm. casein per day	Diet 621 + 20 gm. casein per day	Diet 621 + 60 gm. casein and 50 gm. meat per day
	Mixture Vuj, 0 day	Mixture VujN, 0 day	Mixture VujN, 10 days	Mixture VujN, 20 days	Mixture VujN, 30 days	Mixture Vuj, 35 days	Mixture VujN, 20 days	Mixture VujN, 20 days	Mixture VujN, 20 days
333	340 (18)	265 (13)	145 (7)	95 (5)			170 (10)	235 (13)	325 (17)
530	360 (20)	220 (11)	140 (7)	95 (5)	120 (7)	195 (13)	110 (7)	150 (9)	265 (14)
535	355 (20)	280 (15)	170 (9)	105 (6)	165 (10)	380 (22)		260 (15)	290 (15)

\* The figures in parentheses represent the per cent of the dose excreted.

urinary nor plasma levels showed a consistent increase in retention of the racemic mixture.

When the dogs were fed 10 gm. of casein per day for 20 days, with no increase in weight and very little increase in serum protein level, the excretion of amino acids after infusion of the natural mixture was the same as that found after withholding protein for 10 or 30 days. After feeding 20 gm. of casein per day for 20 days, however, there was definite improvement in weight and serum protein levels, and the increased urinary loss of amino acids after infusion indicated that the protein reserves had been replaced. After consuming the high protein diet, the dogs recovered completely and, when infused with the natural mixture the amino acid content of the urine was slightly greater than when the dogs were fed the stock diet.

After administration of about 2 gm. of nitrogen in the form of amino

acid mixtures, the total nitrogen in the urine generally increased about 1 gm. over that excreted by the fasting dogs before dosing. While the dogs were maintained on the stock diet, the urea nitrogen made up 70 to 80 per cent and the ammonia nitrogen about 5 per cent of the total nitrogen whether the dogs were dosed, fasted, or fed. When the protein intake had been restricted for 30 days the 24 hour fasting excretion of nitrogen decreased to 0.71 gm., of which only 40 per cent was urea nitrogen and 20 per cent was in the form of ammonia. Typical data have been summarized in Table VI.

TABLE VI

*Nitrogen Analyses of Urines of Dogs Given Mixture VujN (Averages, in Gm. of N Per 24 Hours)*

	Stock diet				Protein-free diet					
	Fasted	Fed	Oral	Intra-venous	9th day, fasted	10th day, intra-venous	19th day, fasted	20th day, intra-venous	29th day, fasted	30th day, intra-venous
Dose, N			2.28	2.36		2.17		2.03		1.89
Total "	2.75	8.2	3.71	5.06	1.27	2.24	0.83	1.52	0.71	1.73
Urea "	2.15	6.3	2.59	4.13	0.66	1.32	0.37	0.81	0.31	0.55
NH <sub>3</sub> -N	0.09	0.16	0.25	0.25	0.12	0.13	0.09	0.23	0.14	0.54
$\alpha$ -NH <sub>2</sub> -N	0.02	0.07	0.09	0.26	0.014	0.15	0.015	0.10	0.015	0.14
Protein-free diet + 10 gm. casein daily					Protein-free diet + 20 gm. casein daily		Protein-free diet + 60 gm. casein and 50 gm. meat daily			
	19th day, fasted		20th day, intravenous		19th day, fasted		20th day, intravenous		19th day, fasted	
Dose, N			1.96				2.06			
Total "	1.2		1.36		0.94		2.06		3.76	
Urea "	0.66		0.77		0.45		1.40		2.63	
NH <sub>3</sub> -N	0.14		0.069		0.26		0.23		0.19	
$\alpha$ -NH <sub>2</sub> -N	0.015		0.14		0.015		0.22		0.024	

Microbiological assays showed that, although the pattern of the essential amino acids excreted in the urine did not resemble the pattern of amino acids administered (Figs. 1 and 2), it was essentially the same whether the dogs had normal or subnormal protein reserves at the time of infusion. The proportions of lysine, threonine, and histidine were higher in the urine than in the mixtures infused. Arginine was excreted in about the same proportion as it was administered. The remaining six amino acids were found in the urine in smaller proportions than in the mixture. The fractions of histidine, threonine, and lysine which were found in the urine after

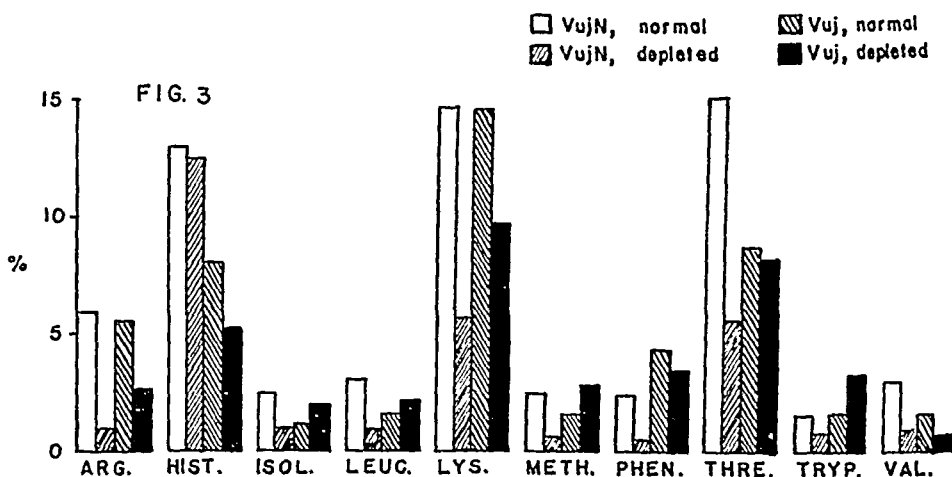
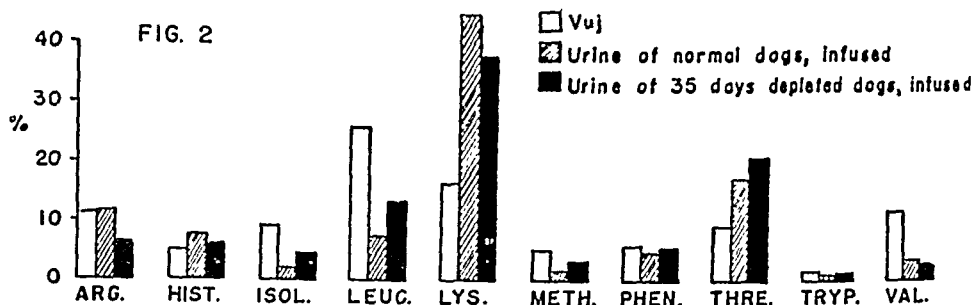
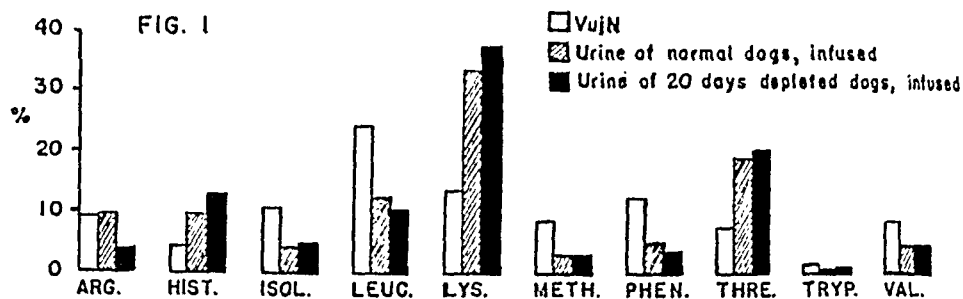


FIG. 1. Essential amino acid pattern in Mixture VujN and in urines after Mixture VujN infusion.

FIG. 2. Essential amino acid pattern in Mixture Vuj and in urines after Mixture Vuj infusion.

FIG. 3. Per cent excretion of individual *l*-amino acids after infusion of normal and depleted dogs.



infusion of the natural mixture ranged between 13 and 15 per cent of the amount administered, whereas only 1.5 to 3 per cent of the tryptophane, leucine, methionine, phenylalanine, isoleucine, and valine was excreted (Fig. 3).

#### DISCUSSION

Rapid infusion of the natural mixture (containing 10 per cent racemic amino acids) into normal dogs resulted in the loss of only 13 per cent of the amino acids in the urine. Since 4 per cent was lost even after oral or slow intravenous administration, the net loss resulting from the increased rate was only 9 per cent.

However, when the racemic mixture (containing 50 per cent racemic amino acids) was infused at the rapid rate (12 mg. of N per kilo per minute), 23 per cent was lost in the urine. At the slow rate (2 mg. of N per kilo per minute) 11 per cent was excreted. Thus, the higher concentration of racemic amino acids apparently was responsible for the urinary loss of 7 to 10 per cent more of the mixture. Calculations based on microbiological assays for the ten essential amino acids indicated that there was a larger proportion of *d* forms in the urines of dogs given this mixture. Since the plasma levels of  $\alpha$ -amino nitrogen were almost identical, whether the infused mixture contained 5 per cent or 25 per cent *d*-amino acids, it is suggested that the increased urinary amino acid excretion in the latter case resulted from a lower kidney threshold for the *d* forms.

It is of interest that the excretion did not vary directly with the rate of infusion, particularly at the higher rates. There was no increase in urinary amino acid when the rate of administration of the natural mixture was increased from 6 to 12 mg. of N per kilo per minute. Furthermore, when the rate of infusion of the racemic mixture was similarly doubled, the loss in the urine increased only slightly, from 19 to 23 per cent.

A state of depleted protein reserves, induced by feeding a diet free of protein, decreased the urinary loss of the natural mixture by 50 per cent without showing any increase in plasma levels, although the decrease in blood volume in such dogs (Weech, Wollstein, and Goettsch (11)) might be expected to cause an increase in plasma levels. It has been reported by Goettsch, Lyttle, Grim, and Dunbar (12) that, when dogs on low protein diets are given amino acid mixtures intravenously, the plasma amino acid levels are elevated as a result of a reduction in the deaminating function of the liver. According to Elman and Heifetz (13), liver damage parallels the decrease in serum protein level in such dogs. However, there is no evidence that the deaminating function of the liver was disturbed in the above experiments.

On the basis of the decreased excretion of the natural mixture by dogs

fed the protein-deficient diet and the slightly increased excretion by dogs fed a high protein diet, it appears that a test of protein reserves might be feasible, with a standardized amino acid mixture and uniform rate of infusion. As in other tests of this type, it is obvious that pathological states which interfere with the normal excretory function of the kidney or utilization of the test material (amino acids) tend to invalidate the results. After feeding dogs the protein-free diet for 35 days, the subsequent feeding of about 1 gm. of casein per kilo per day was insufficient to restore body weights and serum protein levels to normal, and the excretion of amino acids after administration of the natural mixture was no greater than when the protein-free diet was supplied. However, when the daily casein intake was doubled, the animals appeared to be practically normal after 20 days and their excretion of amino acids after infusion of the natural mixture averaged 12 per cent. Before depletion 13 per cent was excreted after infusion of this amino acid mixture at the same rate. When the dogs were infused after consuming the high protein diet, 15 per cent of the administered amino acids was excreted in the urine.

The similarity of the patterns of the essential amino acids in the urines of dogs with normal and subnormal protein reserves after infusion of an amino acid mixture indicates that the dog retains approximately the same combination of amino acids, whether depleted or not. The quite different pattern of the amino acid mixture administered from that of the excretion pattern suggests that the mixture may not be ideal—that the proportion of amino acids in the mixture is not the same as that utilized by the dog, or that certain amino acids are lost in the urine in disproportionately large amounts as a result of differences in kidney clearance (Pitts (14)). If the first hypothesis is correct, lysine, threonine, and histidine are present in excess in the mixture, and others are lacking. However, if the second hypothesis holds true, the mixtures might be altered in the reverse manner to make up for the inability of the kidney to retain these three amino acids.

#### SUMMARY

1. The intravenous infusion of 220 mg. of nitrogen per kilo in the form of an amino acid mixture (VujN), consisting essentially of the *l* forms, to normal dogs at a fast rate (12 mg. of N per kilo per minute) resulted in a loss of 13 per cent in the urine. When the mixture was administered intravenously at one-sixth this rate, or orally, 4 per cent appeared in the urine.

2. When a mixture containing 50 per cent racemic amino acids (Mixture Vuj) was infused at the fast rate, 23 per cent was lost in the urine. At three rates of infusion, 6 to 10 per cent more of this mixture was excreted than after infusion of the mixture which contained only 10 per cent racemic

amino acids. This appeared to be due to a lower kidney threshold for *d*-amino acids.

3. The urinary excretion of amino acids by dogs given an amino acid mixture intravenously decreased 30 to 50 per cent during protein depletion. A daily dietary supplement of 10 gm. of casein to the previously depleted dogs had little, if any, effect on excretion of the amino acid mixture. The urinary excretion of amino acids after infusion of the amino acid mixture was essentially the same whether the dogs were fed the stock diet or 20 gm. of casein per day, but after increasing the protein intake by feeding 60 gm. of casein and 50 gm. of meat daily, the excretion of the amino acid mixture was slightly increased. These results indicate that amino acid excretion after infusion of a standardized amino acid mixture might reflect the state of protein reserves.

4. The pattern of the essential amino acids in the urines of dogs given amino acid mixtures did not resemble the pattern of the mixtures infused, and was not greatly altered by protein depletion.

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# STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

## XIV. ON THE SYNTHESIS OF MERCAPTURIC ACIDS IN MAN\*

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Several species of animals are able to synthesize the corresponding mercapturic acids from ingested halogenated benzenes (2), naphthalene (3), anthracene (4), benzyl chloride (5), and *p*-bromobenzyl bromide (6). The synthesis of the above mercapturic acids was established by the isolation of the acids from the urine of animals and identification of the substances by analysis and comparison with authentic synthetic products.

Circumstantial evidence for the synthesis of the corresponding mercapturic acids in animals from administered phenanthrene (7), benzene (8), diphenyl (9), cholic acid (10), methylcholanthrene, pyrene, and 3,4-benzopyrene (11), and "butter yellow" (12) has also been presented, but, so far, to our knowledge no demonstration of such a synthesis in any animal species has been reported.

The difficulties connected with the administration of these potentially dangerous substances to human beings account for the fact that no direct evidence for the synthesis of mercapturic acids in man has been reported.

We have administered *S-p*-bromophenyl and *S*-benzyl derivatives of cysteine to human subjects and isolated from the urine the corresponding mercapturic acids. Although incomplete for conclusive demonstration of a direct synthesis of the corresponding mercapturic acids from bromobenzene and benzyl chloride, the data furnish the first direct evidence for the synthesis of mercapturic acids in man from the cysteine derivatives of substances which have been demonstrated to yield mercapturic acids in animals.

### EXPERIMENTAL

*Preparation of Cysteine Derivatives*—*S*-Benzyl derivatives of cysteine were prepared by reduction of *L*-cystine in liquid ammonia with metallic

\* A portion of the data was published in a preliminary report (1). The publication of the complete data was delayed by our service overseas during the war.

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sodium, followed by benzylation with benzyl chloride and racemization of the resulting S-benzyl-*l*-cysteine to S-benzyl-*dl*-cysteine. The latter was then resolved into its component isomers (13). S-Benzyl-*dl*-cysteine had a melting point of 155–156° and a 2 per cent solution in 1 *N* NaOH showed no optical activity. S-Benzyl-*d*-cysteine had a melting point of 216–218° and a 1 per cent solution in 1 *N* NaOH had an optical rotation of  $[\alpha]_D^{24} = -24^\circ$ . S-Benzyl-*l*-cysteine also melted at 216–218° and a 1 per cent solution in 1 *N* NaOH had an optical rotation of  $[\alpha]_D^{24} = +23^\circ$ .

*p*-Bromophenyl-*l*-cysteine was prepared from *p*-bromophenylmercapturic acid which was isolated by us in the course of past years from the urine of various animals. *p*-Bromophenyl-*l*-cysteine had a melting point of 192–193° and a 1 per cent solution in 0.2 *N* NaOH had an optical rotation of  $[\alpha]_D^{24} = +17.3^\circ$ . All the substances were analytically pure, as was indicated by analysis for carbon, hydrogen, nitrogen, sulfur, and bromine. The corresponding acetyl derivatives of *p*-bromophenyl-*l*-cysteine and of *dl*, *d*, and *l* isomers of S-benzylcysteine were prepared by acetylation with acetic anhydride. The substances were analytically pure and showed the correct optical rotation. In all cases, the excretion products which were isolated from the urine were analyzed and their properties were compared with those of the synthetic products.

*Feeding Experiments*—The author and a female volunteer were used as subjects in this study. Both subjects were in good health and apparently normal. Each compound was fed with water after a meal on three different occasions to each of the subjects. The urine was collected during the 24 hours following the ingestion of the substance. The author ingested 3 gm. and the other subject 2 gm. of each of the cysteine derivatives in each experiment. No ill effects of any kind were observed objectively or subjectively upon ingestion of any of the substances. Upon ingestion of S-benzylcysteine derivatives, a mild stinging of the mucous membranes of the mouth and a somewhat unpleasant odor persisted for about 5 hours. Both subjects were on an ordinary mixed diet which was apparently free from benzoic acid. Upon completion of the feeding experiment with each substance, a week's interval was allowed before proceeding with the next feeding.

*Isolation Procedure*—In general, the procedure for the isolation of the excretion products of S-benzylcysteine was as follows: The 24 hour sample of urine was made neutral to litmus paper and evaporated under reduced pressure to one-fourth its volume, cooled, and the pH adjusted to 7. The separated material was removed by filtration, treated with dilute ammonia, centrifuged to remove insoluble matter, and the supernatant liquid was decolorized with a small amount of norit<sup>1</sup> and filtered. The filtrate was

<sup>1</sup> Mercapturic acids are readily adsorbed on norit, from which they can be eluted with ethanol. Decolorizing carbon in the isolation procedures involving acetylated

exactly neutralized with dilute hydrochloric acid. The separated material was recrystallized three times from hot water. In this manner, S-benzyl-*d*-cysteine was isolated from the urine.

The filtrate from the material which separated at pH 7 was acidified with HCl (strong acid to Congo red paper) and extracted with either ethyl ether or ethyl acetate. Satisfactory results were obtained with either solvent. The ether or ethyl acetate extract was evaporated to dryness under reduced pressure, and the residue was dissolved in boiling water, decolorized with a small amount of norit, and filtered hot. On cooling, the acetyl derivatives separated. On acidification of the filtrate, hippuric acid contaminated with the acetyl derivatives of S-benzylcysteine separated out. The isolated products were purified by repeated recrystallization from hot water. N-Acetyl-S-benzyl-*l*-cysteine was separated from the *dl* isomer by fractional crystallization of the isolated mixture of the acetyl derivatives from ethanol. Considerable losses of both products were suffered and the yields of these derivatives which are reported below are minimal.

The acetyl derivatives of S-benzylcysteine can be isolated first from the acidified concentrated urine, followed by the isolation of S-benzyl-*d*-cysteine from the neutralized filtrate. Either method was found satisfactory in recovery studies on normal urines to which S-benzylcysteine, *d*- or *l*-, and all the isomers of the acetyl derivative were added together or separately.

After feeding *p*-bromophenyl-*l*-cysteine, *p*-bromophenylmercapturic acid was isolated from the concentrated urine by the chloroform extraction procedure of McGuinn and Sherwin (15).

### Results

3.0 gm. of S-benzyl-*l*-cysteine were ingested and 1.2 gm. of N-acetyl-S-benzyl-*l*-cysteine were isolated from the urine. The substance was analytically pure and proved to be identical with the synthetic material, as is indicated by the analytical data shown below.

	C	H	N	S	Acetyl	M.p. °C.	$[\alpha]_D^{25}$
Synthetic $C_{17}H_{19}SNO_2$ .	56.95	5.93	5.54	12.65	16.98	143-144	-44
Found.	56.78	6.01	5.64	12.33	16.75	143	-43

Similar results were obtained in five additional experiments on the two subjects.

3.0 gm. of S-benzyl-*d*-cysteine were ingested. 200 mg. of N-acetyl-S-benzyl-*l*-cysteine, 480 mg. of N-acetyl-S-benzyl-*dl*-cysteine, 460 mg. of S-benzyl-*d*-cysteine, and 160 mg. of hippuric acid were isolated from the

amino acid derivatives should be used judiciously. Lawrie (14) employed "Merck's medicinal charcoal" for the isolation of *p*-iodophenylmercapturic acid from rat and rabbit urine.

urine. In the analytical data on the isolated material (a) represents N-acetyl-S-benzyl-*l*-cysteine, (b) N-acetyl-S-benzyl-*dl*-cysteine, (c) S-benzyl-*d*-cysteine, and (d) hippuric acid.

	C	H	N	S	Acetyl	M.p. °C.	$[\alpha]_D^{25}$
(a) Synthetic $C_{12}H_{11}SNO_2$ .	56.95	5.93	5.54	12.65	16.98	144	-44
Found.	56.90	5.82	5.43	12.40	16.73	143	-43
(b) Synthetic $C_{12}H_{11}SNO_2$ .	56.95	5.93	5.54	12.65	16.98	155-156	0
Found.	56.87	5.81	5.53	12.55	16.45	155-157	0
(c) Synthetic $C_{10}H_{11}SNO_2$ .	56.87	6.16	6.64	15.16		216-218	-24
Found.	56.73	6.00	6.60	15.00		216-217	-24
(d) Synthetic $C_9H_7NO_2$ .	60.04	5.03	7.82			187	
Found.	59.74	5.26	7.65			187	

Similar results were obtained on repeated administration of S-benzyl-*d*-cysteine to both subjects.

3.0 gm. of S-benzyl-*dl*-cysteine were ingested. The qualitative results were similar to those obtained with S-benzyl-*d*-cysteine, except that no hippuric acid could be isolated. The latter might have been present in the urine, but it escaped our detection. The analytical data were similar to those shown above.

3.0 gm. of *p*-bromophenyl-*l*-cysteine were ingested and 1.3 gm. of pure N-acetyl-*p*-bromophenyl-*l*-cysteine were isolated from the urine. The substance was identical with *p*-bromophenylmercapturic acid which was excreted in the urine of various animals upon ingestion of bromobenzene.

	C	H	N	S	Acetyl	Br	M.p. °C.	$[\alpha]_D^{25}$
Synthetic $C_{11}H_{12}SNO_2Br$ .	41.54	3.77	4.40	10.06	13.49	25.16	152	-13
Found.	41.50	3.68	4.43	9.96	13.00	24.98	152	-12

Similar results were obtained in five additional experiments on the two subjects.

#### DISCUSSION

The data presented above demonstrate the acetylation of *p*-bromophenyl-*l*-cysteine and of *d* or *l* isomers of S-benzylcysteine in man. It is also apparent from the data that the *d* isomer of S-benzylcysteine is less readily acetylated than its enantiomorph, as is indicated by the excretion of unchanged S-benzyl-*d*-cysteine upon its administration. At no time were we able to detect either the unchanged S-benzyl-*l*-cysteine or its optical antipode in a free or acetylated form upon administration of S-benzyl-*l*-cysteine. Only the acetylated S-benzyl-*l*-cysteine could be isolated from the urine. Either our methods of isolation are inadequate for the detection of these substances in the urine, or S-benzyl-*l*-cysteine is completely acetylated in



the amounts fed and is not converted *in vivo* to its enantiomorph. The excretion of N-acetyl-S-benzyl-*l*-cysteine upon the administration of *d*- or *dl*-S-benzylcysteine indicates inversion of the *d* derivative to the acetylated optical antipode. Whether this inversion occurred via the mechanism proposed by Knoop and Blanco (16), as we suggested earlier (5) and as was reaffirmed later (17), is a subject for discussion from which we wish to abstain for the present, pending direct evidence *pro* or *contra*. The acetylation, however, of *p*-bromophenyl-*l*-cysteine and of *d* or *l* isomers of S-benzylcysteine in man, appears to be direct, as we concluded earlier in our experiments with *p*-bromophenyl-*l*-cysteine in the rat (18).

In discussing the possible mechanisms of acetylation in the dog, we assumed that the mechanism of acetylation in the case of mercapturic acids is not the same as that involved in the acetylation of certain aromatic amines and their derivatives, since the direct acetylation of certain aromatic compounds in which the amino group is attached to the ring could not be shown to take place in the dog (19). In the case of man, on the other hand, Muenzen, Cerecedo, and Sherwin (20) were the first to show direct acetylation of *m*- and *p*-aminobenzoic acid, and Bernhard (21) has now demonstrated that acetic acid participates directly in this reaction. Our experiments demonstrate the acetylation of certain cysteine derivatives in man. For lack of data to the contrary, there appears to be no reason to assume the existence in man of different mechanisms of acetylation for the cysteine derivatives and for aromatic amino acids mentioned above.

#### SUMMARY

1. *p*-Bromophenyl-*l*-cysteine and *d*-, *dl*-, and *l*-S-benzylcysteine were administered to human subjects and the corresponding mercapturic acids were isolated from the urine.

2. S-Benzyl-*d*-cysteine is less readily acetylated than the *l* isomer, although in man both isomers are directly acetylated.

3. There appears to be no valid reason to assume a mechanism of acetylation of these cysteine derivatives in man, different from that supported by experimental evidence; namely, direct acetylation.

Grateful acknowledgment is made to Mr. J. Alicino for the microanalytical work.

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# PHOTOMETRIC DETERMINATION OF TOTAL CHOLESTEROL IN PLASMA OR SERUM BY A MODIFIED LIEBERMANN- BURCHARD REACTION

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The wide-spread use of photoelectric colorimeters in the field of quantitative clinical chemistry has brought with it the realization that many of the visual colorimetric methods in use are grossly inaccurate and that their use may be justified only on the basis of an arbitrary range of normal values for the given procedure. This is particularly true in the case of total cholesterol values which have a reported normal range from about 100 to 400 mg. per cent. The principal sources of the inaccuracies for the cholesterol methods employing the Liebermann-Burchard reaction are well known and have been recently discussed in some detail in a paper by Sperry and Brand (1). They will be mentioned here only because in the procedure presented by the authors the experimental studies of the Liebermann-Burchard reaction were run under conditions somewhat different from those of most other investigators (2-4).

Instead of the usual organic solvents, *e.g.* chloroform, alcohol-ether, alcohol-acetone, etc., used for extracting cholesterol and cholesterol esters from biological fluids, the authors have followed the lead of Drekter (5) who used acetic anhydride as the medium for the simultaneous precipitation of the proteins and the extraction of the total cholesterol present in these fluids. Because of the extreme rapidity of development of the maximal color density, pure acetic anhydride was unsuited for the photometric determination of total cholesterol. It was found experimentally that a mixture of acetic anhydride and 1,4-dioxane (3:2 by volume) not only would extract cholesterol and cholesterol esters from biological fluids with the simultaneous precipitation of proteins, but in addition would reduce the rate of development of maximal color density to a point at which the Liebermann-Burchard reaction might be used to obtain accurate photometric readings.

The method presented here is unlike any other found in the literature in that no attempt is made to remove the water from the biological fluid;

\* A major portion of this problem was completed overseas while the authors were members of the Chemistry Section of the Seventh Medical Laboratory of the Third United States Army.

instead, an amount of water equivalent to that present in the biological fluid is added to the cholesterol standards. In the course of the procedure, the water present in the standard or the unknown is converted into acetic acid when the tube containing the water and acetic anhydride is heated in a boiling water bath for a specified time. It is a known experimental fact that the Liebermann-Burchard reaction takes place in acetic acid solution as well as in chloroform, although the rate of reaction may vary with the different solvents (6).

Many investigators have shown that the rate of reaction for pure cholesterol with the Liebermann-Burchard reaction is much slower than that for a corresponding amount of cholesterol ester (as total cholesterol). It is mainly for this reason that the more reliable methods for total cholesterol require the conversion of the cholesterol ester present into the alcohol form previous to the addition of the colorimetric reagents. However, for the routine clinical analysis of total cholesterol in biological fluids it would be most desirable if this step could be omitted, provided that it was accomplished without any corresponding reduction in the accuracy of the procedure. This problem was solved by developing a *new* total cholesterol standard composed of equal parts of cholesterol and cholesterol ester which are dissolved in a solution of acetic anhydride-dioxane (3:2). Since this standard solution is 50 per cent cholesterol ester, it more closely approximates the actual conditions found in most sera or plasma. Moreover, in the setting up of the standard curve, these standards are run (with the addition of an equivalent amount of water, heating, filtration, etc.) in exactly the same manner as are the unknowns. Since a valid objection might be raised with respect to the variability of the ratio of cholesterol esters to total cholesterol as found in biological fluids, separate studies were made for both pure cholesterol and cholesterol esters in acetic anhydride-dioxane solution with the Liebermann-Burchard reaction. Although cholesterol ester develops color at a somewhat higher rate than does a corresponding amount of pure cholesterol, the maximal density values reached are about the same for both. Furthermore, these maximal values remain constant over a sufficient time interval so that various mixtures of cholesterol and cholesterol ester *can* be determined, *without* conversion of the esters into the alcohol form, from the same curve. This experimental work also indicates the importance of using the maximal density value rather than a given time interval for the photometric reading if consistent results are to be obtained.

In agreement with Sperry and Brand (1) it has been found that relatively slight changes in temperature produce significant changes in the maximal density and in the rate at which those values are reached. In this procedure the temperature is rigidly controlled at 37.5°, which is a

higher temperature than that advocated by most other investigators. The reason for this is that the addition of dioxane to the acetic anhydride reduces the rate at which the green color develops in the Liebermann-Burchard reaction to such a marked degree that a higher temperature is required to obtain the maximal color density within a reasonable time interval.

Many investigators (1, 7) have stressed the advantage of such experimental factors as the mixing of acetic anhydride and sulfuric acid previous to its addition to a chloroform or acetic acid solution of total cholesterol, the control of the temperature at which the acetic anhydride and sulfuric acid are mixed and added to the total cholesterol solution, the need for developing the color in total darkness, etc. In the method as given in this paper, although the concentrated sulfuric acid is added directly from a micro burette to the total cholesterol solution at room temperature and no special precautions are taken to allow the color development to take place in complete darkness, highly reproducible results have been obtained for standards, unknowns, and recoveries of added cholesterol and cholesterol ester. The fact that no special precautions are required for the addition of the Liebermann-Burchard reagents or for the color development, except for temperature control, further enhances the simplicity of this method compared to other procedures.

#### EXPERIMENTAL

##### *A. Reagents and Apparatus—*

1. Standard cholesterol solution. Dissolve 0.1000 gm. of pure cholesterol in 100 ml. of pure dry chloroform in a 100 ml. volumetric flask. (1 ml. is equivalent to 1.00 mg. of cholesterol.)

2. Dilute cholesterol standard. Dilute the above standard 1:5 with chloroform in a 100 ml. volumetric flask. (1 ml. is equivalent to 0.20 mg. of cholesterol.)

3. Standard cholesterol ester solution. Dissolve 0.1105 gm. of pure cholesteryl acetate (Eastman Kodak, No. 2391) in 100 ml. of pure, dry chloroform in a 100 ml. volumetric flask. (1 ml. is equivalent to 1.00 mg. of total cholesterol.)

4. Dilute cholesterol ester standard. Dilute the above standard 1:5 with chloroform in a volumetric flask. (1 ml. is equivalent to 0.20 mg. of total cholesterol.)

5. Standard cholesterol solution (new). Weigh 25 mg. of pure dry c.p. cholesterol and 27.7 mg. of cholesteryl acetate into a 500 ml. Erlenmeyer flask. Add 240 ml. of acetic anhydride-dioxane solution; heat the flask for approximately 15 minutes in a boiling water bath until all the solid is in solution. Cool to room temperature and wipe the outside of the flask to remove any moisture. Transfer the solution quantitatively to a 250 ml.

volumetric flask and dilute to mark with acetic anhydride-dioxane solution. Mix and transfer to a clean, dry, glass-stoppered, brown bottle. (1 ml. is equivalent to 0.20 mg. of total cholesterol.)

6. Acetic anhydride-dioxane solution. Mix 3 volumes of reagent grade acetic anhydride with 2 volumes of 1,4-dioxane (Eastman Kodak, No. P2144). Transfer to a clean, dry, glass-stoppered, brown bottle. The mixture is stable in the absence of moisture. Dioxane vapor is toxic and all work with this reagent should be done in a hood.

7. Sulfuric acid, concentrated, reagent grade.

8. Micro burette, 2.00 or 5.00 ml., graduated in 0.05 ml. or in smaller divisions.

9. Photoelectric colorimeter, Evelyn type, with selected Pyrex test-tubes graduated at 5 and 10 ml.

All reagents used in this procedure should be of reagent grade and free from water. All glassware used should be chemically clean and dry.

*B. Selection of Color Filter*—0.20 mg. and 0.50 mg. of total cholesterol standards were prepared, as described under the preparation of the standard curve in (D) below, and the data for spectral transmission for the modified Liebermann-Burchard reaction were obtained with a Coleman Junior spectrophotometer. The readings were taken at room temperature and between 25 and 35 minutes after the addition of the colorimetric reagents. The data obtained are illustrated in Fig. 1 and show a minimum transmittance at about 650  $\mu$ . This wave-length corresponds approximately to a combination of Corning glass Filters 241, 4 mm., and 397, 2 mm., for filter type photometers.

### *C. Studies of Factors Affecting Color Development with Modified Liebermann-Burchard Reaction*

*1. Effect of Variation of Amount of Acetic Acid from Water. Preparation of Samples*—To two sets of graduated colorimeter tubes, each containing five tubes, were added 1.00 ml. (0.20 mg.) and 3.00 ml. (0.60 mg.) of the dilute cholesterol standard in chloroform, respectively. The tubes were placed in a boiling water bath and the solvent evaporated off until no odor of chloroform was detectable. To each pair of standards, one containing 0.20 mg. and the other 0.60 mg. of cholesterol, were added the various amounts of water shown in Fig. 2. The contents of each tube were diluted to the 5.00 ml. mark with the acetic anhydride-dioxane solution; the tubes were heated in a boiling water bath for 30 minutes and then cooled to room temperature.

*Development of Color*—At this point each pair of tubes was handled separately. To the first tube was added 0.25 ml. of concentrated sulfuric acid from a micro burette. Immediately after, an interval timer which had

previously been set for 5 minutes was started up. The contents of the tube were well mixed, care being taken to see that no unmixed acid remained on the sides of the tube. The tube was then left in a rack at room temperature. About  $\frac{1}{2}$  minute before the 5 minute interval elapsed, 0.25 ml. of concentrated sulfuric acid was added to the second tube. At the expira-

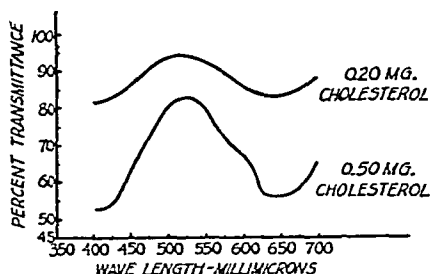


FIG. 1. Spectrophotometric curves for total cholesterol

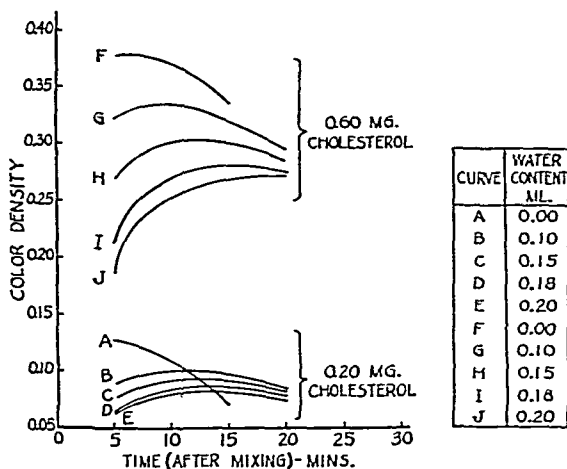


FIG. 2. Effect of variation of acetic acid obtained from water and acetic anhydride on color development.

tion of the first 5 minute interval, the timer was reset for the same interval and the first tube was placed in a water bath maintained at  $37.5^{\circ}$ . The contents of the second tube were then well mixed and the tube allowed to remain at room temperature. At expiration of the second 5 minute interval, the timer was again reset, the second tube was placed in the  $37.5^{\circ}$

water bath, and the first tube was removed, wiped dry, and read in the photoelectric colorimeter with a  $650\text{ m}\mu$  filter against a reagent blank set at 100 per cent transmission. In this manner a series of readings was taken at 5 minute intervals until a maximal density value was reached, when the readings began to increase again. The second tube was read exactly 5 minutes after the first one. All other pairs of tubes were run exactly in the same manner.

The variation of density values with time for cholesterol standards containing various amounts of water is shown in Fig. 2. From these data the following conclusions may be drawn: that the presence of small amounts of water has the greatest proportional effect on decreasing the rate of color development as well as the maximal density value for a fixed amount of cholesterol; that, as the water content increases, the further addition of water causes progressively smaller changes in the rate of reaction and in the maximal density value for a fixed amount of cholesterol.

These experimental data prove that, if accurate results are to be obtained, the volume of water added to the standards, to compensate for the amount present in serum or plasma, should be maintained within fairly narrow limits. Since it was intended to use a 0.20 ml. sample in running the unknowns, and since 90 per cent of serum or plasma is actually water, 0.18 ml. of water was added to each tube in performing all the subsequent experimental runs and in setting up the standard curves.

2. *Effect of Varying Ratio of Acetic Anhydride to Dioxane*—The samples were prepared in the manner described above except that 0.50 and 1.00 mg. of cholesterol standard were used and 0.18 ml. of water was added to each tube, the final volume being the same. The color was developed as above.

The maximal density obtained in these runs was plotted against the various acetic anhydride to dioxane ratios for the two cholesterol standards and the curves obtained are shown in Fig. 3. These results indicate that the maximal density increases continuously as the acetic anhydride to dioxane ratio increases for a given cholesterol value. It is possible to obtain almost any density value desired for a fixed amount of cholesterol by choosing the proper ratio of acetic anhydride to dioxane. A value of 3 volumes of acetic anhydride to 2 volumes of dioxane was chosen for two reasons. First, because the maximal density values for standards ranging from 0.20 to 1.00 mg. of cholesterol fall somewhere between 20 and 80 per cent transmission, which is the most desirable range for colorimetry. The second reason is shown in Fig. 4, which gives the variation of density values with time for the various acetic anhydride to dioxane ratios. The density values for the 3:2 ratio reach a maximum in about 15 minutes and there is only a slight variation in the maximal value between the 15 and 25 minute intervals.



3. *Effect of Variation of Temperature and Time of Reading*—The standards for this run were prepared exactly as given above except that a constant volume of 0.18 ml. of distilled water was added to each tube.

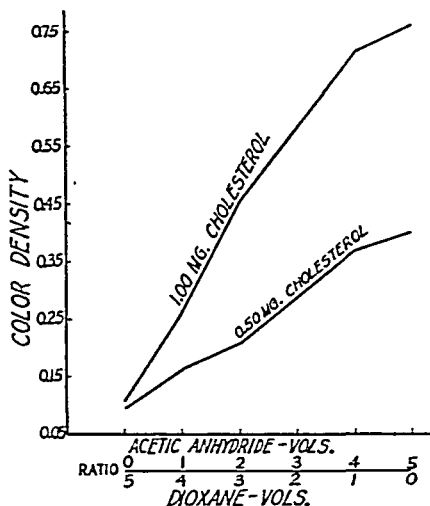


FIG. 3. Effect of variation of ratio of acetic anhydride to dioxane on maximal density value.

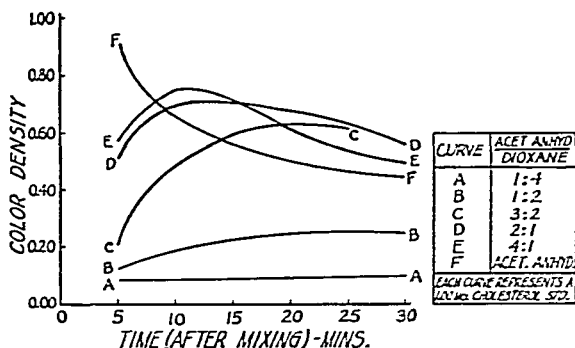


FIG. 4. Variation of transmission values with time for varying acetic anhydride ratios.

The color was developed exactly as described above except that instead of the tubes being placed in a bath at only one temperature (37.5°), the

color development took place in baths maintained at the various temperatures shown in Fig. 5.

Fig. 5 shows the relationship between the density values obtained for a given cholesterol value and the time of reading at the various temperatures at which color is developed. From the results shown in Fig. 5, it can be concluded (1) that as the temperature increases the time at which the maximal density is reached decreases; (2) that as the temperature increases the maximal density value obtained with a fixed amount of cholesterol increases; (3) that for a constant temperature, the variation of the density value at  $\pm 5$  minutes from the maximal density value is about  $\pm 1$  scale division of the colorimeter.

In agreement with the results of many previous investigators, the data

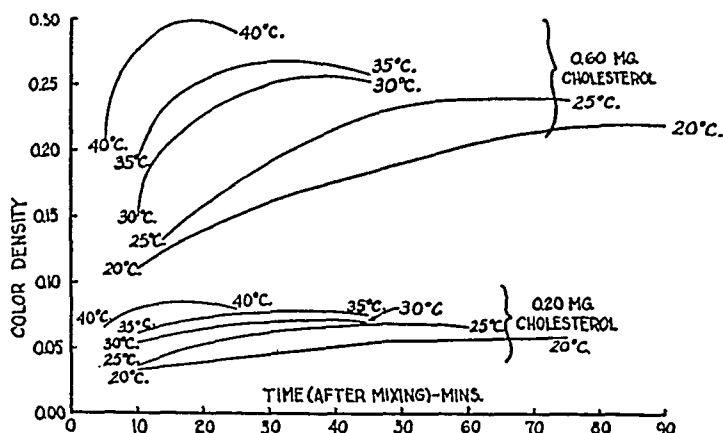


FIG. 5. Effect of variation of temperature and time of reading on color development of cholesterol with the modified Liebermann-Burchard reaction.

obtained show the need for rigid temperature control if accurate cholesterol values are to be obtained. The temperature of  $37.5^{\circ}$  was chosen because it is a conveniently available laboratory temperature, and because the maximal color at this temperature develops somewhere between 15 and 20 minutes; so that the time required for each determination is considerably reduced. However, it should be pointed out that any other constant temperature below  $40^{\circ}$  may be used provided that both standards and unknowns are run at the same temperature.

4. *Effect of Temperature at Which Colorimetric Reagents Are Mixed*—Sperry and Brand (1), Shapiro *et al.* (7), and other investigators lay stress on the technique employed in adding the acetic anhydride-sulfuric acid mixture to the chloroform solution of cholesterol as a potential source of

error in the Liebermann-Burchard reaction. Shapiro *et al.* have recommended using a mixture of acetic anhydride and sulfuric acid prepared at room temperature to avoid any errors due to the difficulty of measuring accurately small quantities of sulfuric acid and to reduce the temperature changes due to variable heating effects. Sperry and Brand carry this procedure a step further and recommend that the reagents be mixed in an ice bath and maintained at that temperature until added to the cholesterol solution in chloroform. Since the matter is still controversial, and since it would be desirable to avoid the temperature effect of adding a solution at 0° to one at the bath temperature, this problem was investigated for the acetic anhydride-dioxane-water solution of cholesterol.

A series of standards was prepared containing 0.20 and 0.60 mg. of cholesterol in the manner given above which included the addition of the

TABLE I  
*Effect of Temperature of Mixing Reagents on Color Development*

Temperature of mixing	Total cholesterol mg.	Color density (Evelyn colorimeter)			
		15 min.	20 min.	25 min.	30 min.
Ice bath, 0°	0.20	0.080	0.088	0.092*	0.091
	0.60	0.258	0.284	0.292*	0.288
Water, 15°	0.20	0.080	0.081	0.089*	0.086
	0.60	0.268	0.288	0.290*	0.278
Room temperature, 19.5°	0.20	0.088	0.092*	0.092	0.089
	0.60	0.276	0.292*	0.292	0.278

\* Maximal density value.

0.18 ml. of distilled water and the use of the 3:2 solution of acetic anhydride-dioxane.

Each pair of standards was adjusted to the temperatures given in Table I previous to the addition of the concentrated sulfuric acid from the microburette. The rest of the procedure is exactly as given above.

The maximal density values obtained in this run, as is shown in Table I, prove that the temperature of the solution at the time of addition of the concentrated sulfuric acid has no effect on the maximal density value obtained and that the acid may be added at *room temperature*. The maximal temperature developed by the addition of concentrated sulfuric acid to the acetic anhydride-dioxane-water solution of cholesterol was about 32.5° for the color development; the heat generated in this step does not affect the color development.

5. *Effect of Varying Time of Heating Acetic Anhydride-Dioxane-Water Solution of Cholesterol*—A series of 1.00 mg. of cholesterol standards was

prepared exactly as given above, including the addition of 0.18 ml. of distilled water and the use of the 3:2 acetic anhydride-dioxane solution, except that the tubes were heated for 15, 30, and 45 minutes in the boiling water bath.

Development of color was carried out by the usual procedure.

The results obtained in this run are given in Table II and show that continuing to heat the tubes in a boiling water bath for more than 15 minutes has a negligible effect on the color development.

*D. Preparation of Standard Curve for Cholesterol*—The experimental data obtained above indicate that reproducible cholesterol values can be obtained only if rigid control is maintained over such factors as the temperature of color development, ratio of acetic anhydride to dioxane, amount of water present in the sample, and if the maximal density value rather than a specific time interval is used for the colorimetric readings. Less control

TABLE II  
*Effect of Variation of Time of Heating*

Cholesterol standard, 1.00 mg.

Time of heating  <i>min.</i>	Color density			
	10 min.	15 min.	20 min.	25 min.
15	0.409	0.456	0.462*	0.456
30	0.447	0.472*	0.469	0.447
45	0.414	0.456*	0.456	0.435

\* Maximal density value.

is required over such factors as the temperature at which the colorimetric reagents are mixed and the time of heating the tubes in the boiling water bath. These data were then applied to the preparation of a standard curve for pure cholesterol.

*Preparation of Samples*—Into a series of graduated colorimeter tubes were pipetted, in duplicate, amounts of the cholesterol standards in chloroform equivalent to 0.20, 0.40, 0.60, 0.80, 1.00, and 1.20 mg. of cholesterol. The rest of the procedure was run exactly as given under (C, 2) except that the 3:2 acetic anhydride-dioxane solution was used for all the samples.

Color was developed by the usual procedure.

The curve obtained by plotting the average maximal density values against the cholesterol standards is shown in Fig. 6.

*E. Effect of Variation of Temperature and Time of Reading on Values for Cholesterol Ester*—Since many investigators have called attention to the more rapid rate of color development with cholesterol esters compared to

pure cholesterol in chloroform, or as found experimentally, in acetic acid solution, it was decided to investigate this matter for acetic anhydride-

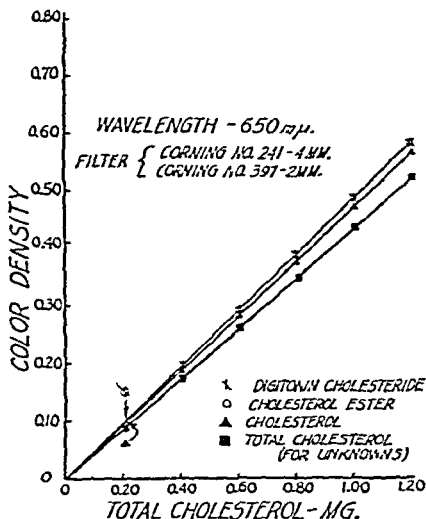


Fig. 6. Photometric curves for cholesterol, cholesterol ester, total cholesterol (as digitonin cholesterolide), and total cholesterol (for unknowns).

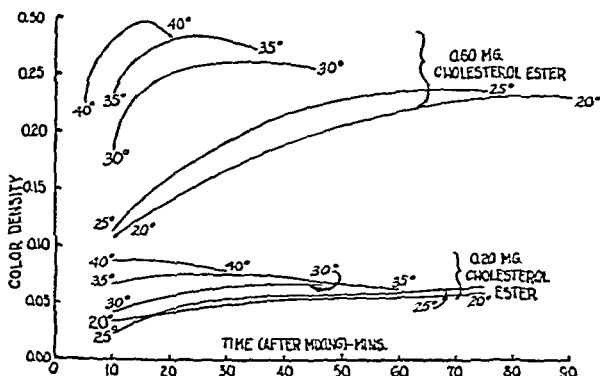


Fig. 7. Effect of variation of temperature and time of reading on color development of cholesterol ester with modified Liebermann-Burchard reaction.

dioxane-water solutions of cholesterol. It was assumed that there would not be any marked difference in the results obtained with cholesterol ester

standards for factors such as the ratio of acetic anhydride to dioxane, the amount of water present, the temperature at which the colorimetric reagents are added, or the time of heating the tubes in the boiling water bath; therefore these factors were not reinvestigated for cholesterol ester. However, it was considered desirable to reinvestigate for cholesterol ester such factors as the effect of the variation of temperature at which color development takes place, and the time of reading the samples upon the maximal density values obtained for a fixed amount of the standard (calculated as total cholesterol). From these data it could be ascertained whether, at any particular temperature or reading time, the same density values could be obtained for cholesterol ester as for an equivalent amount of cholesterol, thus avoiding the necessity of converting the ester into the alcohol form to obtain accurate results for the total cholesterol value.

The run was performed exactly in the same manner as is given under (C, 3) for pure cholesterol except that cholesterol ester (cholesteryl acetate) standards in chloroform corresponding to 0.20 and 0.60 mg. of total cholesterol were used. The results obtained in this run are shown in Fig. 7.

It is concluded from these experimental data that the effect of temperature on the maximal density values is exactly the same as with pure cholesterol. A comparison of Figs. 5 and 7 leads to an even more important conclusion; namely, that for the most part the maximal density values for *both* cholesterol and cholesterol ester fall within the same time interval, and if the temperature remains constant, the maximal density values for a constant amount of cholesterol ester are practically *identical* with those obtained for an equivalent amount of pure cholesterol.

*F. Preparation of Standard Curve for Cholesterol Esters*—This was prepared in the same manner as the curve for pure cholesterol, except that cholesterol ester standards containing the equivalent amount of total cholesterol were used. The results obtained for the average maximal density values are plotted against the total cholesterol values, and the curve obtained is illustrated in Fig. 6. The data indicate that, while the two curves are *not* identical, they are sufficiently close together, particularly in the lower range of values, where the large majority of unknowns will fall, to warrant the use of a common curve for both cholesterol and cholesterol esters, thus avoiding the involved process of converting one form into the other, previous to the color development.

*G. Determination of Maximal Density Values for Known Mixtures of Cholesterol and Cholesterol Ester*—As further proof that the use of a common curve for both cholesterol and cholesterol ester is warranted in the acetic anhydride-dioxane-water procedure, a series of known mixtures of cholesterol and cholesterol ester was prepared in such ratios as are likely to occur in pathological human sera or plasma. These mixtures were

then run exactly in the same manner as is described for the cholesterol standards in (D). The maximal density values obtained for these various known mixtures are given in Table III together with the average maximal density values taken from the cholesterol and cholesterol ester curves in Fig. 6.

The data in Table III prove that the results obtained for these mixtures are generally within  $\pm 0.02$  mg. of total cholesterol of the theoretical value taken from the curve and furnish conclusive evidence of the validity of employing a single curve for the determination of the total cholesterol

TABLE III

*Determination of Total Cholesterol Content of Known Mixtures of Cholesterol and Cholesterol Ester*

Cholesterol added	Cholesterol ester added	Total cholesterol added	Ratio, cholesterol ester to total cholesterol	Color density			Average color density from curve	Average error
				15 min.	20 min.	25 min.		
mg.	mg.	mg.	per cent					per cent
0.50	0.50	1.00	50	0.456	0.462*	0.450	0.469	-1.50
0.50	0.50	1.00	50	0.459	0.462*	0.453	0.469	-1.50
0.70	0.30	1.00	30	0.435	0.453*	0.453	0.469	-3.42
0.70	0.30	1.00	30	0.438	0.456*	0.456	0.469	-2.77
0.30	0.70	1.00	70	0.401	0.447*	0.447	0.469	-4.70
0.30	0.70	1.00	70	0.423	0.465*	0.459	0.469	-0.85
0.50	0.20	0.70	23.5	0.328	0.335*	0.325	0.330	+1.52
0.50	0.20	0.70	23.5	0.328	0.330*	0.323	0.330	$\pm 0.00$
0.20	0.50	0.70	71.4	0.319	0.321*	0.312	0.330	-2.73
0.70	0.50	1.20	41.7	0.542	0.557*	0.553	0.569	-2.11
0.70	0.50	1.20	41.7	0.534	0.549*	0.542	0.569	-3.52
0.50	0.70	1.20	58.2	0.549	0.553*	0.538	0.569	-2.81

\* Maximal density.

content of an unknown sample regardless of the ratio of cholesterol to cholesterol ester present.

*H. Method for Photometric Determination of Total Cholesterol in Serum or Plasma*—Into test-tubes (150  $\times$  15 mm.) containing approximately 5 ml. of acetic anhydride-dioxane solution was pipetted, in duplicate, 0.20 ml. of clear, unhemolyzed serum or plasma. The contents were mixed well by shaking, care being taken that no unmixed serum or plasma remained on the sides of the tube. The tubes were heated in a boiling water bath for 30 minutes with occasional shaking, and the moisture was wiped off the outside. The contents were filtered through Whatman No. 42 filter paper (7 cm.) into graduated colorimeter tubes. The original tubes were washed with 0.25 ml. portions of acetic anhydride-dioxane solution, these washings

being poured through the filter paper until the total volume of the filtrate in each tube was 5.00 ml.

The development of color was carried out exactly as given under (C, 1).

The average maximal density value obtained by this procedure is used to find the total cholesterol value from the standard curve prepared as given in the following section.

*I. Preparation of Standard Curve for Use with Unknown Samples*—In order to overcome the effect of the slight variation noted above between the curves for pure cholesterol and cholesterol ester and to prepare a total cholesterol curve which more closely simulated the actual conditions found in the case of human sera or plasma, the new cholesterol standard which has already been described under (A) was used in this run. In addition, while it was not desirable to go through the tedious processes of filtration, washing, making up to volume, etc., in establishing the theoretical concepts of the method, it was desirable that these processes be included in the preparation of a standard curve for actual use with unknown samples, so that any changes in the density values due to these additional steps would be compensated for.

Into a series of the test-tubes ( $150 \times 15$  mm.) were pipetted, in duplicate, amounts of the new cholesterol standard equivalent to 0.20, 0.40, 0.60, 0.80, and 1.00 mg. of total cholesterol. The total volume in each tube was then brought up to about 5 ml. with acetic anhydride-dioxane solution. 0.18 ml. of distilled water was added to each tube and the contents well mixed so that no unmixed water remained on the sides of the tube. The remainder of the procedure for the unknown samples was carried out exactly as described under (H).

Color was developed by the usual procedure.

*J. Determination of Total Cholesterol by Visual Colorimetry*—If a photoelectric colorimeter is not available, the new cholesterol standard can also be used for visual colorimetry and is prepared for the color development exactly as described under (I) above, with 2.00 and 4.00 ml. aliquots of the cholesterol standard corresponding to 0.40 and 0.80 mg. of total cholesterol, respectively. The unknown samples of serum or plasma are prepared simultaneously for the color development exactly as described for the photoelectric method in (H) above. The color development is performed by the usual procedure except that the reading of the unknown against the standard is performed in the visual colorimeter between the 15 to 20 minute time interval since, as is shown in Table IV, the maximal density values usually fall in this interval for serum or plasma.

*K. Determination of Normal Serum Values and Recoveries of Added Cholesterol*—Some 50 samples of clear, unhemolyzed sera, sent in for serological examination, were analyzed by the procedure as given under (H).



A typical set of results, to illustrate the importance of using the maximal density value rather than a constant time interval, is given in Table IV.

TABLE IV

*Photometric Determination of Total Cholesterol Content of Normal Human Serum and Recoveries of Added Cholesterol, Cholesterol Ester, and Mixtures of Both*

Serum No.	Color density			Total cholesterol in serum	Total cholesterol added	Total cholesterol recovered	Standard used	Error
	10 min.	15 min.	20 min.	mg.	mg.	mg.		per cent
1	0.141	0.163*	0.166	0.53			New†	
1	0.246	0.250*	0.242	0.57	0.20	0.19	"	-5.0
1	0.282	0.301	0.305*	0.90	0.50	0.52	"	+4.0
2	0.166	0.168*	0.163	0.38			"	
2	0.235	0.252*	0.250	0.57	0.20	0.21	"	+5.0
2	0.317	0.337*	0.330	0.76	0.40	0.38	"	-5.0
3	0.194	0.196*	0.187	0.44			"	
3	0.276	0.282*	0.270	0.64	0.20	0.20	"	±0.0
3	0.367	0.372*	0.364	0.84	0.40	0.40	"	±0.0
4	0.194*	0.194	0.180	0.44			"	
4	0.238	0.268*	0.260	0.61	0.20	0.17	"	-8.5
4	0.357	0.364*	0.347	0.83	0.40	0.39	"	-2.5
5	0.187	0.192*	0.181	0.43			"	
5	0.262	0.282*	0.276	0.63	0.20	0.20	"	±0.0
5	0.332	0.347*	0.342	0.79	0.40	0.36	"	-10.0
6	0.187	0.194*	0.189	0.44			"	
6	0.260	0.282*	0.278	0.63	0.20	0.19	"	-5.0
6	0.347	0.369*	0.364	0.84	0.40	0.40	"	±0.0
7	0.189	0.197*	0.192	0.44			"	
7	0.260	0.276*	0.276	0.62	0.20	0.18	"	-10.0
7	0.337	0.364*	0.359	0.83	0.40	0.39	"	-2.5
8	0.163	0.174*	0.174	0.39			"	
8	0.204	0.237	0.244*	0.46	0.20	0.17	"	-15.0
8	0.328	0.347*	0.347	0.79	0.40	0.40	"	±0.0
9	0.187	0.194*	0.187	0.44			"	
9	0.260	0.276	0.284*	0.64	0.20	0.20	Cholesterol	±0.0
9	0.272	0.335	0.367*	0.84	0.40	0.40	"	±0.0
10	0.132	0.160	0.163*	0.38			"	
10	0.276	0.332	0.359*	0.70	0.40	0.42	Cholesterol	+5.0
10	0.420	0.495	0.523*	1.18	0.80	0.80	ester	±0.0

\* Maximal density value.

† The new standard is 50 per cent cholesterol and 50 per cent cholesterol ester.

Recoveries of total cholesterol added to sera, including that added as pure cholesterol, cholesterol ester, and mixtures of both (new standard), are also included in Table IV.

*L. Preparation of Total Cholesterol Standard Curve from Digitonin Choles-*

*teride*—Into a series of 15 ml. graduated centrifuge tubes were pipetted, in duplicate, amounts of the pure cholesterol standard in chloroform ranging from 0.20 to 1.00 mg. of total cholesterol. The solvent was evaporated off by placing the tubes in a boiling water bath until no odor of chloroform could be detected. 3 ml. of alcohol-acetone (1:1) solution were added to each tube and the cholesterol dissolved by warming the tubes gently in the water bath. The cholesterol was then converted into digitonin cholesterolide by the procedure of Schoenheimer and Sperry (8). To the pure, dry digitonin cholesterolide precipitate contained in the centrifuge tubes were added 0.18 ml. of distilled water and 5 ml. of the acetic anhydride-dioxane solution. The tubes were heated for 30 minutes in a boiling water bath and the precipitate dissolved with the aid of occasional stirring with a glass rod placed in each tube. The tubes were removed from the bath and the contents filtered into the graduated colorimeter tubes through Whatman No. 42 filter paper (7 cm.). The centrifuge tubes were washed with 0.25 ml. portions of acetic anhydride-dioxane, these washings being poured through the filter paper until the total volume of the filtrate in each tube was 5.00 ml.

The development of color was carried out exactly as described under (C, 1).

The average maximal density values in this run were plotted against the cholesterol values and the curve obtained is shown in Fig. 6. This curve is practically identical with that of the cholesterol ester standard curve.

*M. Comparison of New Procedure with Digitonin Cholesterolide Procedure for Normal Sera*—The following experiments were intended mainly to furnish additional proof of the reliability of the new procedure and to explore the possibility of using it for the separate determination of cholesterol and cholesterol esters. (1) The total cholesterol content of a number of normal sera was determined exactly as given in (H) above. (2) An alcohol-acetone (1:1) extract of the same sera was prepared as for the Schoenheimer-Sperry (8) method and the total cholesterol content determined as follows:

A 5.00 ml. aliquot of the alcohol-acetone filtrate of the sera (1:25 dilution) was pipetted into a test-tube (150 × 15 mm.) and the contents evaporated just to dryness, care being taken not to char the residue in this step. The residue was dissolved in a few ml. of chloroform and the contents again evaporated just to dryness. The chloroform evaporation was repeated once again, but this time the heating was continued until no further odor of chloroform could be detected. 0.18 ml. of distilled water and 5 ml. of acetic anhydride-dioxane solution were added to each tube. The tubes were then heated in a boiling water bath for 30 minutes with occasional shaking, removed from the bath, etc., the remainder of the procedure being

performed exactly as described under (H) above. The results for this run are given in Table V.

TABLE V

*Comparison of New Procedure with Digitonin Cholesteride Procedure and Studies of Effect of Time of Standing of Sample on Total Cholesterol Value*

Tube No.	Serum or plasma No.	Color density						Total cholesterol mg. per cent	Method used
		5 min.	10 min.	15 min.	20 min.	25 min.	30 min.		
1	11	0.135	0.163	0.177*	0.176	0.169	0.163	200	New
2	11	0.155	0.182	0.183*	0.181	0.171	0.153	205	5 ml. alcohol-acetone (1:1) extract, evaporated with CHCl <sub>3</sub>
3	11	0.066	0.085	0.086*	0.086	0.082		216	Digitonin cholesteride, 3 ml.
4	12	0.147	0.181	0.184*	0.176	0.163	0.155	205	New
5	12	0.138	0.176*	0.174	0.163	0.155	0.146	210	Same as (2)
6	12	0.061	0.080	0.085*	0.082	0.080		216	" " (3)
7	13	0.115	0.155	0.166*	0.166	0.160	0.152	190	New
8	13	0.141	0.168*	0.168	0.157	0.149	0.138	190	Same as (2)
9	13	0.068	0.082*	0.078	0.076	0.069		204	" " (3)
10	14	0.174	0.229	0.235*	0.227	0.215	0.199	265	Freshly drawn normal plasma; new
11	14	0.149	0.218	0.237*	0.233	0.224	0.208	270	Same plasma 1 hr. after drawing; new
12	14	0.204	0.238	0.242*	0.229	0.220		275	Same plasma 9 hrs. after drawing; new
13	14	0.172	0.218	0.227*	0.222	0.208	0.191	260	Plasma from blood after standing 1 hr.; new
14	14	0.174	0.222	0.229*	0.222	0.203	0.191	260	Plasma from blood 9 hrs. after standing; new

\* Each value is the average of two or more determinations.

The total cholesterol content of the alcohol-acetone filtrate of the same sera as above was then determined, in duplicate, by the Schoenheimer-Sperry method (8), except that instead of dissolving the digitonin cholesteride in concentrated acetic acid, the precipitates were treated exactly as were the standards in (L). The results obtained in this run are also given in Table V.

The remaining data in Table V were all obtained with the new procedure and tend to confirm the findings of Kraus and Kalal (9) that the total cholesterol content of human serum or plasma does *not* change on standing, and that these determinations need *not* be done immediately after the blood is drawn.

#### DISCUSSION

The results obtained in the studies of the effect of the variation of the amount of acetic acid derived from water on the color development with the modified Liebermann-Burchard reaction show the decided effect which even traces of moisture have on the maximal density values obtained and on the time in which these values are reached for a fixed amount of cholesterol. Unless all water is completely removed from the unknown in the treatment of the sample previous to the color development, it is to be expected that the standard being made up in the pure solvent will develop more color in the same time interval than will the unknown, and thus low results will be obtained. In the procedure presented in this paper, the small amounts of acetic acid derived from water present both in the standards and in the unknowns actually have a beneficial effect on the method. Its presence prolongs the time in which maximal color is reached, allowing the use of higher bath temperatures; it stabilizes the color formation so that accurate readings of the maximal density value may be obtained over a 10 minute or longer interval; and the presence of traces of moisture, in the reagents and on the glassware, does not have the same detrimental effect on color formation as in the more commonly used procedures.

Several different samples of acetic anhydride and dioxane from different manufacturers were used. While the maximal density values obtained for a constant amount of cholesterol were about the same for acetic anhydride-dioxane solutions obtained from different sources, the time in which this value was reached varied markedly with different products. It was for this reason as well as for the greater reproducibility of values that the maximal density reading, rather than the time after mixing, was selected as the criterion for the total cholesterol values. The importance of this factor has also been stressed in a recent paper by Clarke and Marney (10) which deals with a modification, for use with the photoelectric colorimeter, of the Schoenheimer-Sperry method. Since the acetic anhydride-dioxane solution is stable over long periods of time, it is best to prepare a batch of the reagent large enough to set up all the necessary standard curves and time and temperature studies, and to have enough left over for a large number of unknowns.

In these experiments, identical results were obtained whether standards were run in a dark incubator with a thermostatic control, a water bath in a lighted room, or in an incubator heated by carbon filament bulbs.

The results obtained for normal human sera and plasma (Tables IV and V) by the new procedure indicate that while some of the recoveries of added total cholesterol are somewhat low the maximal error obtained for the recovery of 0.20 mg. of cholesterol is  $-15$  per cent and the average error for all the recovery values is about  $\pm 5$  per cent. They further indicate that good results are obtained for the recoveries whether the total cholesterol is added in the pure form, as the ester, or as a mixture of both forms. Results given in Table V prove that the same values are obtained by the acetic anhydride-dioxane-water procedure whether the extraction of the serum is performed directly or whether the cholesterol is first extracted with an alcohol-acetone (1:1) solution. These latter results are important when both cholesterol and cholesterol ester determinations are contemplated. The final and most conclusive proof of the validity of the method is that it gives results which check experimentally with those obtained by the digitonin cholesterolide method if these latter values are read from a digitonin cholesterolide curve.

The 50 normal sera used in these determinations were obtained from the serology department of this laboratory and the total cholesterol content was determined in from 4 to 48 hours after the blood had been centrifuged and the serum removed. This meant that in some instances the serum was removed almost immediately after clotting had occurred and in other instances only after serum had been standing in contact with the blood clot for 10 to 12 hours. Kraus and Kalal (9) have shown that even under these conditions the total cholesterol values do not change, and the values shown in Table V substantiate their findings and justify the inclusion of these results for serum as normal findings. Considering the random method by which these normal sera were selected, the most surprising thing about the values obtained is the relatively small range which they cover, *i.e.* from about 190 to 270 mg. per cent, and that the large majority of the values falls at about 210 mg. per cent. Although this range may be considered representative for male adults from 20 to 35 years of age, many more determinations will have to be run by the procedure for other segments of the population before it can be stated that this smaller normal range is due to the better reproducibility and greater accuracy of the method compared to other procedures.

There has been some scepticism concerning the reliability of the usual clinical laboratory tests for cholesterol and cholesterol esters and of their value in diagnostic work (11, 12). While it is generally conceded (11) that the Schoenheimer-Sperry (8) method or some of its modifications (13, 10) are the most reliable procedures, their very complexity makes them unsuited for routine clinical work. This investigation was started because the many requests for cholesterol determinations from hospitals in the Third United States Army area, in cases such as infectious jaundice, acute

epidemic hepatitis, etc., indicated a need for such a procedure. Since the method presented in this paper is simple, highly reproducible, and gives results of the same order of accuracy as does the Schoenheimer-Sperry method, the authors believe that it will satisfy the need for a good clinical method for the photometric determination of total cholesterol.

#### SUMMARY

A simple, reproducible, and accurate method is presented for the photometric or visual determination of total cholesterol in human sera or plasma.

The principle of the method depends upon the experimental fact that when a mixture of serum or plasma and acetic anhydride-dioxane (3:2 by volume) is heated in a boiling water bath for 30 minutes there occur simultaneously an extraction of cholesterol and cholesterol esters, precipitation of proteins, and conversion of water present into acetic acid.

A new total cholesterol standard consisting of a solution of equal parts of cholesterol and cholesterol ester in acetic anhydride-dioxane is used in this procedure. In the preparation of the standard curve an amount of water is added to the standards equivalent to that contained in the serum or plasma.

Extensive experimental studies were made of all the factors affecting the color produced with this modified Liebermann-Burchard reaction, including variations in the amount of water present, temperature, time of reading, ratio of acetic anhydride to dioxane, time of heating, method of adding reagents, and ratio of cholesterol to cholesterol ester. These studies furnished conclusive proof that by the use of this procedure conversion of the cholesterol ester into cholesterol previous to the addition of the Liebermann-Burchard reagents is not required for accurate results.

Some 50 sera obtained from normal male adults were examined by this procedure and gave a normal range from about 190 to 270 mg. per cent of cholesterol, with the large majority of the values falling at about 210 mg. per cent. Recoveries of total cholesterol added as cholesterol, cholesterol ester, or a mixture of both, averaged about  $\pm 5$  per cent, and the serum values by this procedure checked with those obtained with a digitonin cholesteride method.

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# THE EFFECTS OF INSULIN IN FLUORIDE-POISONED RATS

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In a previous study (1) it was found that the administration to rabbits of fluoride, cyanide, azide, iodoacetate, and malonate, all known inhibitors of carbohydrate metabolism *in vitro*, uniformly resulted in markedly elevated blood concentrations of glucose, lactic acid, and phosphate. Insulin was found to prevent the rise in blood glucose concentration without affecting the behavior of the other measured blood components. It seemed of interest to study the variations in liver and muscle glycogen under these conditions in the hope that the mechanism by which insulin prevented the rise in blood sugar concentration might be elucidated.

## EXPERIMENTAL

Male rats of the Vanderbilt strain (2) weighing over 250 gm. were used in all experiments. Sodium fluoride was the inhibitor chosen because of the smooth and uniform behavior of rabbits when given this substance. In a series of preliminary trials it was found that when this drug was given at a level of 250 mg. per kilo, subcutaneously, most animals died between 120 and 180 minutes later. The insulin dosage was 80 units per kilo, subcutaneously. The rats given insulin alone (Group 3) were sacrificed 150 minutes later. Groups 4 and 5 were given sodium fluoride 30 minutes after administration of the insulin. Group 5 also received 1.5 gm. of glucose per rat intraperitoneally simultaneously with the insulin administration. All rats were well fed on a standard commercial chow.

Blood glucose was determined by a modification of the micro technique of Folin (3), liver and muscle glycogen by the procedure of Good, Kramer, and Somogyi (4), and blood lactic acid was estimated in pooled samples of blood, according to the method of Barker and Summerson (5). The results are summarized in Table I. Each value is the mean of a group of six animals.

As in the rabbit, sodium fluoride poisoning induced a marked elevation in blood glucose and lactic acid concentrations. At the same time, liver and muscle glycogen were seriously depleted. Data obtained from only a few animals indicated that in the rat, as in the rabbit, fluoride poisoning elicited a marked elevation of the serum phosphate concentration and this was not prevented by insulin. From the data previously obtained in rabbits (1)

it may be presumed that fatal poisoning by other inhibitors of carbohydrate metabolism, *e.g.* malonate, cyanide, iodoacetate, would also result in depletion of liver and muscle glycogen. It is of some interest to note that such depletion of muscle glycogen, *in vivo*, has only been seen heretofore in association with convulsive activity.

While not indicated in Table I, it should be stated that the lower terminal blood sugar values were obtained from those animals with the lowest liver glycogen concentrations. This has been interpreted to mean that liver glycogen is the source of the blood glucose of fluoride-poisoned animals, as it is in normal animals. This conclusion was also apparent from a separate study wherein both well nourished and fasted rats were given fluoride in the usual manner and blood samples were collected for glucose determinations every 30 minutes. The results are summarized as Curves A and B re-

TABLE I

*Effects of Insulin on Chemical Changes in Fluoride Poisoning*

Each value represents the mean of a group of six rats.

Group No.	Drug	Blood glucose	Blood lactic acid	Liver glycogen	Muscle glycogen
		mg. per cent	mg. per cent	per cent	per cent
1	None	116 $\pm$ 12	16 $\pm$ 3	3.9 $\pm$ 0.5	0.71 $\pm$ 0.05
2	NaF	202 $\pm$ 26	193 $\pm$ 25	0.63 $\pm$ 0.08	0.14 $\pm$ 0.02
3	Insulin	41 $\pm$ 7	24 $\pm$ 5	4.8 $\pm$ 0.4	0.68 $\pm$ 0.07
4	Insulin + NaF	91 $\pm$ 8	214 $\pm$ 17	0.82 $\pm$ 0.10	0.60 $\pm$ 0.04
5	Insulin + NaF + glucose	116 $\pm$ 9	167 $\pm$ 18	1.21 $\pm$ 0.09	0.63 $\pm$ 0.05

spectively in Fig. 1. Although the terminal values were never as high as the peak values in either group, the well nourished animals were invariably hyperglycemic throughout the experiment, while the fasted rats, with smaller initial glycogen reserves, were hypoglycemic when they died. Thus, the terminal hypoglycemia reported by Kaplan and Greenberg (6) in fluoride-poisoned rats which had previously been fasted was due to depletion of the liver glycogen and probably not, as they suggested, to fluoride inhibition of the liver phosphatase which hydrolyzes glucose-6-phosphate.

Insulin, given alone, lowered the blood sugar in the usual fashion, while the values for liver and muscle glycogen and blood lactic acid were not significantly different from the untreated controls. When insulin-treated rats were poisoned with fluoride, the terminal blood sugar values were essentially normal, as had previously been observed in rabbits. However, the lactic acid concentration was elevated and the liver glycogen depleted much as when fluoride was given alone, while the muscle glycogen concentration was only slightly diminished.

A separate group of animals was given a large dose of anterior pituitary extract (25 mg. per 100 gm. subcutaneously in saline) 30 minutes before fluoride administration, but this had little effect except to provide somewhat higher terminal blood glucose values.

#### DISCUSSION

Since the actual mechanisms underlying the events observed during fluoride poisoning are, at best, incompletely understood, it is not possible at this time to interpret adequately the effects of insulin in this connection. However, a few conclusions seem to be warranted. The rise in blood glucose concentration in fluoride poisoning appears to be due to an accel-

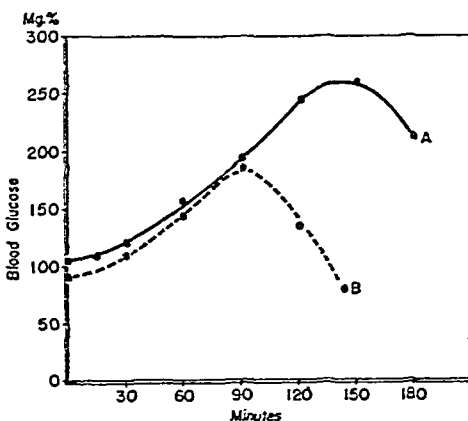


FIG. 1. Blood sugar concentrations during sodium fluoride poisoning in well nourished (Curve A) and fasted rats (Curve B).

erated "glycogenolysis" in the liver. However, the lactic acid which accumulates in the circulation may arise in the liver as well as the peripheral tissues. Since the liver glycogen was depleted in the insulin-fluoride-treated animals despite the essentially normal blood glucose levels, insulin does not appear to inhibit, *in vivo*, any of the various steps involved in liver glycogenolysis; *i.e.*, glycogen  $\rightarrow$  glucose-1-phosphate  $\rightarrow$  glucose-6-phosphate  $\rightarrow$  glucose + inorganic phosphate. Under the conditions prevailing in fluoride poisoning insulin appeared to have no effect upon the formation of lactic acid or its removal either by oxidation or glyconeogenesis.

The only action of insulin in this system which suggests itself and seems compatible with all the data is an acceleration of the conversion of blood glucose to muscle (extrahepatic) glycogen or some intermediary metabolite in that process. This would account for two otherwise anomalous situa-

tions, *i.e.* that the excessive hepatic glycogenolysis was not inhibited by insulin while the blood glucose remained essentially normal, and also that the conversion of peripheral glycogen to lactic acid was unaffected by insulin; yet the muscle glycogen concentration was scarcely diminished as compared with the effect of fluoride alone. Thus if the rate of glycolysis in liver and muscle and the rate of hepatic glycogenolysis were unaffected by insulin while the conversion of blood glucose to muscle glycogen (or some intermediate such as glucose-6-phosphate) was accelerated to a rate commensurate with lactic acid synthesis, thereby sparing the muscle glycogen, the present data would appear to be rational. The possibility of an accelerated hepatic glycogenesis as well is not excluded by these data.

This hypothesis is in agreement with the accelerated disappearance of blood glucose effected by insulin in the hepatectomized dog (7) and the increased muscle glycogen resulting from the administration of insulin plus glucose as compared with giving glucose alone (8). The conversion of blood glucose to tissue glycogen involves three steps, *i.e.*, glucose + adenosine triphosphate  $\rightarrow$  glucose-6-phosphate  $\rightarrow$  glucose-1-phosphate  $\rightarrow$  glycogen. The data reported herein do not permit any deduction as to which of these reactions is actually accelerated by insulin. However, since the present work was done, this point has been clearly established by Price, Cori, and Colowick (9), who demonstrated that insulin overcomes the inhibiting effect of anterior pituitary extract on the action of hexokinase, the enzyme which catalyzes the formation of glucose-6-phosphate in the scheme above.

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#### SUMMARY

Fatal sodium fluoride poisoning in rats was accompanied by markedly elevated blood glucose and lactic acid concentrations and severely diminished liver and muscle glycogen concentrations. Insulin, given 30 minutes before the fluoride, prevented the increase in blood glucose and decrease in muscle glycogen concentration, but did not affect the accumulation of lactic acid or the depletion of liver glycogen. It is concluded that, under these conditions, insulin operated by accelerating the conversion of blood glucose to muscle glycogen or some intermediary metabolite, presumably glucose-6-phosphate.

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# THE CARBOXYLASE ACTIVITY OF JACK BEANS (*CANAVALLIA ENSIFORMIS*) AND SOY BEANS (*GLYCINE HISPIDA*)

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The determination of urea, according to the method of Krebs and Henseleit (1), involves the manometric measurement of the carbon dioxide formed by the action of urease at an acid pH. In the course of an investigation on urea synthesis in liver slices it was observed that very high "urea" values were obtained when pyruvate and oxalacetate were added as substrates. Further study revealed the presence of a potent carboxylase system in the jack bean meal. Soy bean meal, on the other hand, proved to be practically free of carboxylase activity. Dickens and Weil-Malherbe (2) have also noted the carboxylase activity of jack bean meal.

Experiments are here reported which demonstrate the presence of a potent carboxylase system in jack bean meal and the effect of this system on the manometric determination of urea in biological systems.

## EXPERIMENTAL

*Preparation of Jack Bean and Soy Bean Meal Extracts*—Aqueous extracts of commercial jack bean meal (Arlco) and freshly ground soy bean meal were prepared according to Krebs and Henseleit (1). 10 gm. of meal were suspended in 35 ml. of H<sub>2</sub>O and the suspension allowed to stand overnight at room temperature. The suspension was then centrifuged and the centrifugate discarded. The supernatant was brought to pH 5 by the addition of 0.1 volume of 3 M acetic acid-sodium acetate buffer, pH 5, and allowed to stand in the cold for 1 hour. The solution was again centrifuged and the yellow opalescent supernatant used.

*Dialysis of Urease and Carboxylase Solutions*—A portion of the supernatant resulting from centrifuging an aqueous extract of jack bean meal (first step above) was dialyzed in the cold against saturated Na<sub>2</sub>HPO<sub>4</sub> solution for 4 hours and then against distilled water for 15 hours. The dialyzed solution was then brought to pH 5 with acetic acid-sodium acetate buffer. In comparing the dialyzed and non-dialyzed solutions for urease and carboxylase activities, the volumes were adjusted to correct for dilution during dialysis.

*Measurement of Urease and Carboxylase Activity*—Urease activity was measured manometrically in Warburg vessels at 38°. The main compart-

ment contained 1.7 ml. of  $H_2O$ , 1.0 ml. of 0.01 M urea solution plus 0.3 ml. of 3 M acetic acid-sodium acetate buffer, pH 5. The side arm contained 0.5 ml. of the enzyme solution.

Carboxylase activity was measured in a similar manner, except that 1 ml. of 0.01 M pyruvic acid, adjusted to pH 5, was used in place of urea.

### Results

*Effect of Dialysis on Urease and Carboxylase Activity of Jack Bean Meal Extracts*—The effect of dialysis on urease and carboxylase activity of aqueous extracts of jack bean is shown in Fig. 1. It is apparent that

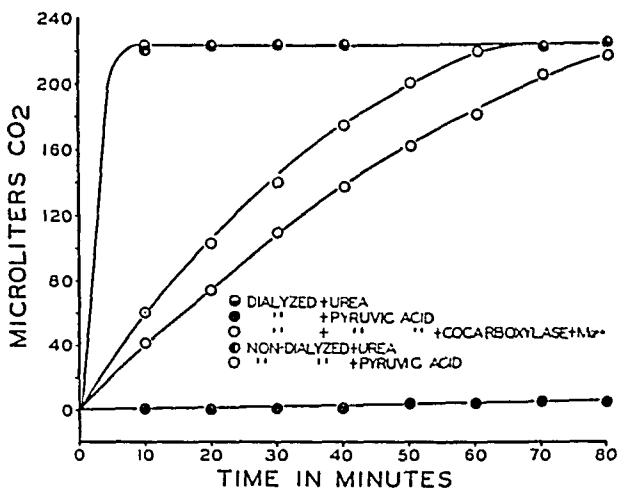


FIG. 1. Effect of dialysis on carboxylase activity of jack bean meal extracts. The experimental conditions are as described in the text. Cocarboxylase addition, 50  $\gamma$ ;  $Mg^{++}$  addition, 2 mg. per cup.

urease activity is unaffected by dialysis. Carboxylase activity, on the other hand, is practically completely lost. The carboxylase system can be reactivated to approximately 95 per cent of its original activity by the addition of cocarboxylase and magnesium ions. This effect definitely proves the presence of a typical carboxylase system in jack bean meal.

As seen from Fig. 1, the carboxylase activity of jack bean meal is considerable. Calculated on a basis of nitrogen content of extracts, jack bean meal has about 40 per cent the activity of brewers' yeast prepared according to the method of Westerkamp (3).

*Comparison of Carboxylase Activity of Jack Bean Meal and Soy Bean Meal*—A comparison of the carboxylase activity of jack bean and soy bean meal revealed that the latter is practically free of carboxylase activity (Fig. 2).



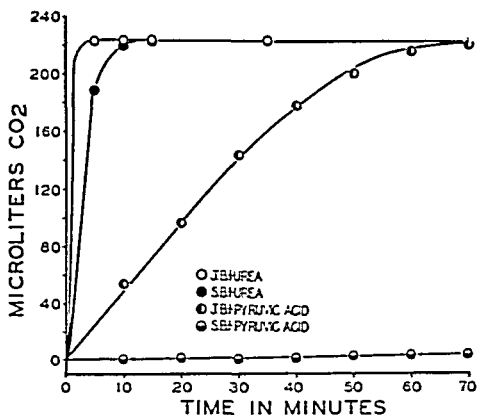


FIG. 2. Comparison of carboxylase activity of jack bean and soy bean meal extracts.

TABLE I

*Urea Formation in Rat Liver Slices, As Measured by Jack Bean Meal Extracts and Purified Urease (Arlco)*

Rat fasted 48 hours. Glucose (200 mg. per cent) and saline bicarbonate medium; gas phase 5 per cent  $\text{CO}_2$ , 95 per cent  $\text{O}_2$ ;  $38^\circ$ ; substrate concentrations,  $\text{NH}_4\text{Cl}$ , 0.0187 M; other substrates, 0.0167 M. Incubation time, 60 minutes.

Substrate	$Q_{\text{urea}}$	
	Arlco urease	Jack bean meal urease
$\text{NH}_4\text{Cl}$ .....	0.67	0.85
Pyruvate .....	1.64	2.05
" and $\text{NH}_4\text{Cl}$ .....	0.44 (7)	12.62 (144)
Oxalacetate .....	1.01 (15)	11.40 (150)
" and $\text{NH}_4\text{Cl}$ .....	3.71 (48)	23.31 (220)
Asparagine .....	3.06 (53)	13.65 (225)
Glutamine .....	3.60	3.18
	3.45	3.68

The figures in parentheses represent microliters of  $\text{CO}_2$  formed. The differences in  $Q$  values are due to differences in tissue weight.

Two crops of soy beans were used. One group of beans was at least 4 years old and failed to germinate. The other beans were of a recent crop and germinated readily. There was no difference in the urease activities of the two crops, and both were free of carboxylase activity.

While soy bean meal extracts can be used as a source of carboxylase-free

urease preparations, it is far more convenient to use a purified commercial preparation. Arlco urease<sup>1</sup> was found to be stable in dry form for long periods of time and to provide highly active urease solutions, completely free of carboxylase, in concentrations of the order of 5 to 10 mg. per ml.

*Use of Jack Bean Meal Extracts for Manometric Urea Estimation in Biological Material*—In Table I typical data from an experiment on urea synthesis in liver are shown. Urea formation was estimated both with an aqueous extract of jack bean meal and with a purified commercial urease preparation (Arlco urease) which was free of carboxylase. It can be seen that in the absence of suitable controls the use of jack bean meal can lead to an erroneous impression as to the rôle of compounds such as pyruvate and oxalacetate on urea synthesis. The suggestion by Leuthardt and Glasson (4) that pyruvate and oxalacetate play intermediate rôles in urea synthesis could be explained, at least from the data published, on this basis. It should be noted (Table I) that even with the use of controls, that is pyruvate and oxalacetate alone, higher urea values could be obtained by having smaller amounts of tissue in the experimental flasks.

In the case of oxalacetate the rate of spontaneous decarboxylation is considerable, even in the absence of carboxylase. In the presence of carboxylase (jack bean meal extracts) very large amounts of carbon dioxide are formed.

*Thiamine Content and Carboxylase Activity of Soy Bean*—The thiamine content of soy beans is listed as 41.0 i.u. per gm. or 12  $\gamma$  per gm. (5). Determination of thiamine in jack bean meal by the thiochrome method<sup>2</sup> gave a value of 9.78  $\gamma$  per gm. Since the jack bean meal represented approximately two-thirds of the whole bean due to previous defatting, this would correspond to a value of about 14.7  $\gamma$  of thiamine per gm. of whole bean. Thus, while the two beans contain approximately the same amount of thiamine, jack beans have an active carboxylase system and soy beans do not.

#### DISCUSSION

The carboxylase activity of plant seeds has not been systematically investigated. Andersson (6) appears to have carried out the first investigation of the enzyme system using pea bean meal. He found that a true carboxylase system was present requiring cocarboxylase and magnesium ions. Zeijlemaker (7) studied the carboxylase system in oat seeds and seedlings and demonstrated an increased activity after the addition of

<sup>1</sup> Purchased from the Arlington Chemical Company.

<sup>2</sup> This determination was carried out through the kindness of Mr. Jack Reinhardt of the Department of Biochemistry.

coccarboxylase. Bunting and James (8) reported the presence of a carboxylase system in barley seedlings.

In the present study, two seeds of the bean family, jack bean (*Canavalia ensiformis*) and soy bean (*Glycine hispida*), reveal striking differences in their carboxylase activity, the former having a potent system and the latter being free of this enzyme. Of considerable interest is the fact that both beans contain approximately the same amount of thiamine. The possibility exists that the need for a carboxylase system in the soy bean is small, due to the high protein and fat and low carbohydrate content. The relatively higher carbohydrate content of other beans of the jack bean type would indicate the requirement of an active carboxylase system for the intermediary metabolism of such beans.

#### SUMMARY

1. Jack beans contain a potent carboxylase system requiring cocarboxylase and magnesium ions.
2. Soy beans contain approximately the same amount of thiamine as jack beans, but are free of carboxylase activity.
3. The use of jack bean meal extracts for the manometric estimation of urea in systems containing pyruvic or oxalacetic acids may lead to erroneous "urea" values, due to the carboxylase activity.

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# ISOLATION OF A PEPTIDE OF *p*-AMINOBENZOIC ACID FROM YEAST\*

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Since Woods (1) showed in 1940 that *p*-aminobenzoic acid (PAB) reverses the bacteriostatic effect of the sulfonamides, the metabolic function of PAB has been the subject of considerable investigation. According to the current view, first advanced by Fildes (2), some essential enzymatic process, in which PAB participates, is blocked by the sulfonamides as the result of a competitive inhibition. We have considered the possibility that a derivative of PAB, elaborated by the cell, might be more directly involved than PAB itself, and have, therefore, undertaken an investigation of the various forms in which PAB occurs in yeast.

Free PAB has been isolated from yeast by Blanchard (3). He suggested the presence also of a bound form, conjugated through the amino group, from which free PAB may be liberated by hydrolytic reactions during autolysis. The present communication is concerned with a second conjugated form of PAB in which the carboxyl group is bound to a strongly acidic polypeptide and the arylamino group is free. It is apparent that this substance may contribute to the increase in diazotizable amine, observed by Blanchard after cell disintegration, merely by becoming available to the diazotizing agent. There is some evidence that in animal tissue also PAB occurs in more than one variety of bound form (4).

The new substance accounts for 20 to 30 per cent of the total PAB content of yeast. Approximately 400 mg. of the purified peptide were obtained from 50 kilos of dried yeast. This represents a yield of about 8 per cent of the peptide present in the first stage of separation. The isolation procedure was necessarily complex and entailed large losses. Details of the procedure are given in the experimental section.

Analytical data indicate that the purified amorphous material is 90 to 95 per cent pure, and that all nitrogenous impurities have been removed. The substance is readily soluble in water, alcohol, and a 1:1 alcohol-ether mixture, but is insoluble in dry ether or acetone. It is very hygroscopic

\* This investigation was supported by grants from the Rockefeller Foundation, the Williams-Waterman Fund of the Research Corporation, and the Lederle Laboratories, Inc. The initial phase of this investigation was supported by the Nutrition Foundation, Inc.

and difficult to free completely from moisture or solvent. Attempts at further purification by ion exchange reagents, such as Amberlite IR-4 or acid-washed alumina, were unrewarding. It was recognized at an early stage that the search for a crystalline derivative would undoubtedly require more material than the few hundred mg. available for study; investigation of structure was, therefore, carried out on material purified by fractionation of the silver, lead, and barium salts and by solvent fractionation.

No attempt has been made to estimate the molecular weight of the peptide. The results indicate that a minimal unit is made up of 1 terminal PAB residue, one amide group, 10 or 11 glutamic acid residues, and 1 unknown amino acid residue, probably acidic in nature.

*Identification of PAB and Mode of Linkage*—The PAB moiety has been identified by isolation from a hydrolysate and conversion to the picryl derivative (see the experimental section). PAB cannot be removed from a solution of the peptide by extraction with ether at pH 3.8 (the isoelectric point of free PAB), except after acid or alkaline hydrolysis. Since the bound PAB responds as an arylamine in the method of Bratton and Marshall (5), only the carboxyl group is involved in a peptide bond. The Bratton and Marshall method has been used also to estimate the PAB content of the peptide, a procedure justified by the fact that the PAB content of *p*-aminobenzoylglutamic acid may be estimated accurately by the same method. Apparently PAB is linked in the molecule in no other form, for the expected amount is recovered after hydrolysis.

*Absorption Spectrum*—Further evidence of the mode of linkage is offered by light absorption data. The position of the absorption maximum of free PAB, as with other benzenoid compounds having ionizing groups, is shifted by a change in pH (Fig. 1, A). At pH 4 the maximum lies at 280  $m\mu$ , while at pH 7 and 13 it appears at 267  $m\mu$ , and at pH 1 absorption is almost completely abolished in this region. Derivatives of PAB in which ionization of the benzenoid carboxyl group is impossible, such as *p*-aminobenzoylglutamic acid (Fig. 1, D) and *p*-aminobenzamide, display a maximum at 275  $m\mu$  which is not altered by changes in pH from 4 to 13, but is abolished at pH 1. The absorption curve of the isolated peptide exhibits the same characteristics as the two latter compounds: (1) a maximum at 270  $m\mu$  in the pH range from 4 to 13 and (2) appreciable diminution at pH 1 (Fig. 1, C). The low absorption at pH 1 is associated with the ionization of the benzenoid amino group. When suppressed, as in N-acetyl-*p*-aminobenzoylglutamic acid (Fig. 1, B), N-acetyl-*p*-aminobenzoic acid, and N-acetyl-*p*-aminobenzamide, the absorption at pH 1 coincides with that observed at higher pH values. This behavior, by contrast to that of the polypeptide, offers further evidence that the arylamino group in the latter is not bound.

The absorption curve of the isolated peptide does not, however, coincide completely with the curve for *p*-aminobenzoylglutamic acid. The extinction coefficient of the former is higher than would be expected, and, though at pH 1 the value is greatly reduced, some residual absorption is observed. An effect such as this might be attributed to acetylation of a fraction of the PAB component. This possibility has, however, been definitely excluded by the facts that (1) the residual absorption is virtually unaltered by mild

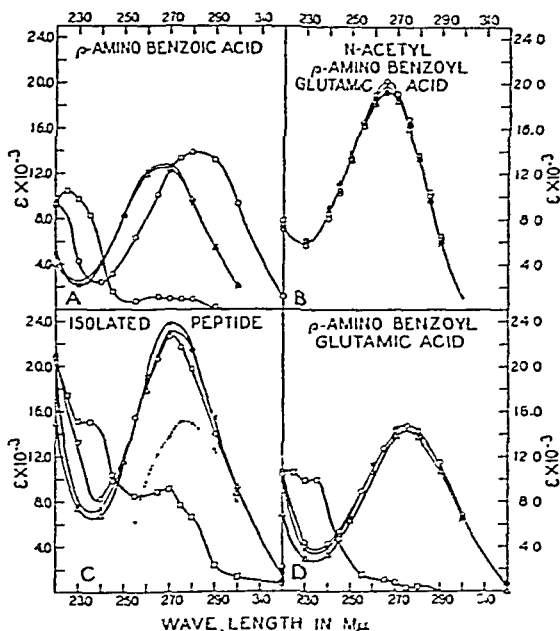


FIG. 1. Light absorption curves of isolated *p*-aminobenzoic acid peptide and related compounds. The data were obtained with buffered solutions (X) at pH 7.0 (0.05 M phosphate); (O) pH 4.0 (0.05 M acetate); and ( $\Delta$ ) in 0.1 N NaOH; ( $\square$ ) 0.1 N HCl. The broken line is the  $\Delta$  curve for the isolated peptide.

hydrolysis (conditions known to remove acetyl groups) and (2) the recovery of PAB on hydrolysis is not greater than expected from its estimated value in the unhydrolyzed peptide. The residual absorption disappears under the conditions employed for complete hydrolysis and cannot be attributed to another light-absorbing constituent of the peptide, since the most likely possibilities, tryptophane, tyrosine, and nucleotides, have been excluded. It is more probably associated with a contaminating pigment, 2 or 3 per cent of which might be sufficient to produce the amount of residual

absorption observed. If it is assumed that the pigment absorption is not affected by pH changes, this absorption may then be subtracted from the curve observed at a higher pH. The resulting curve ( $\Delta$  curve, Fig. 1, C) closely resembles that of the pure peptide; it has a maximum extinction coefficient at 275  $m\mu$  in good agreement with that of *p*-aminobenzoyl-glutamic acid.

*Estimation of Homogeneity*—Three preparations of 42, 159, and 207 mg. have been separately purified and analyzed for the individual components. The analytical data given in Table I show good agreement, considering the experimental error of the methods employed. More convincing evidence of homogeneity is offered by the results of dialysis. The ratio of PAB to total nitrogen was estimated in five successive fractions of the dialysate and was uniform within the experimental error. Certainly short peptides, having a higher ratio of PAB to nitrogen, would have appeared in the first fraction had any been present.

*Estimation of Purity*—Preparations of the purified peptide are ash- and phosphorus-free and contain about 1 per cent carbohydrate. A polypeptide of the composition assigned should contain about 11.6 per cent nitrogen, if the unknown amino acid does not have an unusually long carbon chain. Although data on the nitrogen distribution agree from one preparation to another, the total nitrogen and PAB values, based on dry weights, vary from 10.0 to 10.5 per cent nitrogen and from 7.6 to 8.1 per cent PAB, while the ratio of PAB to nitrogen is the same in all three preparations. The ratios were 1:12.8, 1:12.8, and 1:12.7 respectively. Although the samples were dried at 110° *in vacuo*, there was evidence, from the fact that the largest sample gave the lowest nitrogen value, that the solvent had not been completely removed. The discrepancy cannot be attributed to the method of estimation for, in comparison, Dumas and Kjeldahl values were in close agreement. If attributed entirely to impurities, the material should, at worst, be considered about 90 per cent pure, and, at best, about 95 per cent pure, if the low nitrogen values can be partially explained by incomplete drying of the samples.

*Glutamic Acid Content and Ratio of PAB to Glutamic Acid*—*l*-Glutamic acid is a second component of the polypeptide and accounts largely for the acidic nature of the unhydrolyzed material. It has been isolated as the hydrochloride and estimated by a variety of methods.

Both the Cohen procedure (6), employing chloramine-T, and the sub-micro procedure of Waelsch<sup>1</sup> (7), which depends on the initial use of ninhydrin, respond with *d*- as well as with *l*-glutamic acid. Estimation by bioassay<sup>1</sup> (8) detects only the natural form. Since the results of all three methods are in agreement, the glutamic acid must occur entirely in the

<sup>1</sup> We are greatly indebted to Dr. H. Waelsch and Dr. E. Brand for these analyses.



natural form. The data recorded in Table I show also that each of three preparations gave values in substantial agreement with one another (77.3 to 78.8 per cent glutamic N of total N). In an attempt to decide whether the peptide molecule contains 10 or 11 glutamic acid residues per mole of PAB, the decision must be made on the basis of relatively small differences, as shown by the theoretical values presented in Table I. With few exceptions, submicro analytical methods with 40 to 70  $\gamma$  of material were employed throughout the investigation, owing to the extremely limited supply of peptide. In view of the accuracy of the methods employed and of the purity of the peptide, a final decision is unjustified, although the data appear to favor a 10:1 ratio.

*Components Other Than Glutamic Acid and PAB*—The peptide yields, on acid hydrolysis, 7.0 to 7.9 per cent of its nitrogen as ammonia. As no increase was observed on prolonging the hydrolysis from 11 to 24 hours, the ammonia must be considered to have originated in an amide group.

The sum of the three known constituents, glutamic acid, PAB, and amide nitrogen, accounts for 92 to 93 per cent of the total nitrogen. The remaining nitrogen (7 to 8 per cent) is present as an amino acid, as is shown by the values for  $\alpha$ -amino nitrogen. If the average  $\alpha$ -amino nitrogen value, 84.8 per cent, as determined by estimates of ammonia liberated after treatment with ninhydrin (9), is taken as the basis of calculation, then the sum of this value plus the average amide nitrogen (7.6 per cent) and the PAB nitrogen (7.8 per cent) accounts for the total nitrogen (100.2 per cent) quite well. When  $\alpha$ -amino nitrogen was estimated (10) by the amount of  $\text{CO}_2$  evolved on treatment with ninhydrin, the values were a few per cent higher and were scattered more widely (Table I). It might be inferred from the higher values that aspartic acid, which forms 2 moles of  $\text{CO}_2$ , was perhaps the unknown amino acid.<sup>2</sup> No aspartic acid has been found in the course of attempts at chemical isolation, and bioassay revealed no significant amounts. The higher values may perhaps be attributed to other factors such as the partial decarboxylation of an unknown carboxyl group.

The difference (6.8 per cent) between the glutamic acid nitrogen and the  $\alpha$ -amino nitrogen approximates the value expected (7.1 or 7.7 per cent) on the assumption that 1 amino acid residue other than glutamic acid is present. The unknown amino acid is not identical with any of the following: tryptophane, tyrosine, cysteine, methionine, serine, threonine, arginine, proline, aspartic acid, histidine, lysine, and probably leucine, isoleucine, phenylalanine (see the experimental section); as will be shown below, the substance is probably a dicarboxylic acid.

*Estimation of Carboxyl Groups*—The unhydrolyzed polypeptide has no free amino groups except that attributed to PAB. Electrometric titration

<sup>2</sup> In this case the expected value would be 92.3 or 92.9 per cent.

with alkali shows that pH 7.5 to 8.5 may be taken as the stoichiometric end-point for estimation of the carboxyl groups. No evidence of further buffering was observed in the alkaline region up to pH 11. The titration curve of *p*-aminobenzoylglutamic acid displays an end-point in the same region with exactly 2 equivalents of alkali.

In a polypeptide having one PAB group linked through the carboxyl, 10 or 11 glutamic acid residues, one amide group, and 1 additional amino

TABLE I  
*Analysis of Isolated p-Aminobenzoic Acid (PAB) Peptide*

	Hydrolyzed				Unhydrolyzed	
	Glutamic acid N*	Amide N	Carboxyl N (CO <sub>2</sub> )	$\alpha$ -Amino N (NH <sub>2</sub> )	PAB N	Free carboxyl equivalents
	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>per cent of total N equivalents</i>
Preparation 2	78.8 (C)†				7.7	87.0
“ 3	74.1 “ ‡§	7.7‡ 7.0	84.3‡ 89.8‡ 87.0		7.7	84.5
“ 4	77.3 (B)† 78.7 (W)	7.7   7.9	87.0‡	84.5   85.1	7.8	88.0
Theory for 1 PAB, 1 amide, 10 glutamic, 1 unknown dicarboxylic	76.9	7.7	84.6	84.6	7.7	84.6
Theory for 1 PAB, 1 amide, 11 glutamic, 1 unknown dicarboxylic	78.6	7.1	85.7	85.7	7.1	85.7

\* Glutamic acid was estimated by the Cohen method (C), by bioassay (B), and by the Waelsch method (W).

† Hydrolyzed 24 hours in 5 N NaOH at 106°.

‡ Hydrolyzed 12 hours in 6 N HCl at 106°.

§ There was reason to suspect that the low value of this separately handled sample was due to incomplete hydrolysis.

|| Hydrolyzed 24 hours in 6 N HCl at 106°.

acid residue, the respective numbers of free carboxyl groups arising from glutamic acid residues would be 11/14 (78.6 per cent) or 10/13 (76.9 per cent) of the number of nitrogen atoms. The average value observed, 86 per cent, is appreciably higher and suggests the presence of an extra acidic group (Table I) over that indicated by the above calculations. If the extra carboxyl were associated with the unknown amino acid, the component in question would have to be a dicarboxylic amino acid other than aspartic acid, since this substance has been excluded from consideration.

As was pointed out by Ivanovics and Bruckner (11), with regard to the

polypeptide of *d*-glutamic acid elaborated by *Bacillus subtilis*, the peptide bonds of glutamic polypeptides may involve either the  $\alpha$ -carboxyl or the  $\omega$ -carboxyl groups. Bovarnick (12) has been able to show, by means of racemization studies, that the linkages are of the latter type. In the case of the PAB peptide, the nature of the peptide bonds is as yet unknown.

**Biological Activity**—No biological activity has been observed with the peptide itself with respect to either growth-promoting effects or anti-sulfonamide action, except in so far as the test organism is able to liberate free PAB by hydrolysis. The biological inertness may perhaps be associated with an inability of a molecule of this size to penetrate the cell wall. Several years ago Auhaugen (13) investigated the biological activity of synthetic PAB peptides. He found *p*-aminobenzoyl-*L*-glutamic acid to be 10 times more active than PAB in reversing the bacteriostatic effect of sulfanilamide, but other laboratories have been unable to confirm this observation (14, 15).

#### EXPERIMENTAL

**Isolation and Purification**—The 20-40 strain of Fleischmann's<sup>1</sup> yeast was autolyzed by suspending 1600 gm. in 4800 cc. of water at 40° and incubating the mixture at 38° for 2 hours with frequent stirring. The autolysate was added to 16 liters of water and the pH brought to 2.0 by addition of 800 to 900 cc. of 50 per cent trichloroacetic acid. The resulting precipitate was discarded and the filtrate, which amounted to 16.3 liters, after removal of proteins, contained 69.1 mg. of total PAB. No attempt was made to differentiate between the free and combined forms at this stage, since the subsequent treatment with silver nitrate leaves the free PAB in solution, whereas the silver salt of the PAB-containing polypeptide is soluble in acid solution but insoluble in neutral and slightly acid solution. On the addition of 245 cc. of 25 per cent silver nitrate, acid-insoluble silver salts precipitated out and were discarded. The supernatant, which contained 56.0 mg. of total PAB in a volume of 16 liters, was brought to pH 4.5 with 2 *N* ammonium hydroxide. The resulting precipitate was centrifuged off, washed with dilute silver nitrate, and dissolved in 36 cc. of 50 per cent trichloroacetic acid. To the solution, which had a volume of 142 cc. and contained 13.8 mg. of total PAB, were added 900 cc. of 95 per cent ethyl alcohol. The mixture was allowed to stand at 5° overnight, centrifuged, and the precipitate was discarded after being washed with 100 cc. of 80 per cent alcohol. The combined supernatant and washings contained 12.9 mg. of bound PAB in a volume of 1035 cc. A further purification was effected by conversion to the lead salt which, unlike the silver salt, is insoluble in

<sup>1</sup> Dr. C. Frey of The Fleischmann Company has generously supplied us with large amounts of the yeast.

acidified aqueous alcohol. Accordingly, 175 cc. of 20 per cent lead acetate were added and the precipitate was washed by centrifugation with 200 cc. of water. The crude lead salt was then decomposed by the addition of 20 cc. of 0.5 M phosphate buffer (pH 6.5). After removing lead phosphate, the combined supernatant and washings, containing 6.5 mg. of bound PAB in a volume of 175 cc., were treated with 30 cc. of 25 per cent barium acetate. Water-insoluble barium salts were centrifuged off and extracted twice with 50 cc. portions of hot water. The combined supernatant and washings contained 4.0 mg. of bound PAB in a volume of 300 cc. The crude barium salt, weighing 260 mg., was obtained by precipitation with 2 volumes of ethyl alcohol, and contained 2.0 mg. of bound PAB.

*Purification of Crude Barium Salt*—The chief impurities of the crude material were a pigment, carbohydrate, and a nitrogenous material not containing PAB. Although none of these was acidic, they were carried down to some degree in each precipitation. Effective purification consisted in repeated precipitation of the barium and silver salts followed by precipitation of alcohol-insoluble impurities from solutions of the free peptide, and finally by removal of ether-soluble impurities. The purification was accomplished with a yield of about 50 to 60 per cent when the procedure was controlled by estimations of PAB.

In a typical preparation, 2.5 gm. of crude barium salt were repeatedly extracted with small portions of water, the combined extracts (30 cc.) were made alkaline to phenolphthalein with barium hydroxide, treated with 1 volume of 95 per cent ethyl alcohol, chilled, and the barium salt was centrifuged off. This process of solution and precipitation of the barium salt was repeated until water-insoluble impurities were completely removed. All volumes were kept small for this purpose. The barium salt was then decomposed by the addition of a small excess of dilute sulfuric acid. The solution of the free peptide, containing 16 mg. of PAB in a volume of 30 cc., was converted to silver salt by addition of 5 cc. of 25 per cent silver nitrate and was brought to pH 2 by careful addition of dilute sodium hydroxide. Insoluble material was removed, and the silver salt was precipitated out at pH 6. It was centrifuged off, washed with 1 per cent silver nitrate several times, suspended in  $H_2O$ , and 0.1 N sulfuric acid was added until solution was just achieved. The process of precipitation and solution was repeated until all acid-insoluble silver salts were removed. The silver salt was suspended in about 10 cc. of 50 per cent alcohol and decomposed with  $H_2S$ , in the usual manner. The solution of the free peptide was then brought to a syrup *in vacuo* under a stream of nitrogen and the residue was extracted with 5 cc. of 80 per cent ethyl alcohol. In order to remove all alcohol-insoluble impurities without incurring large losses of the peptide, the process of concentration and extraction was repeated several times with successively

higher concentrations of alcohol until a clear solution of the peptide in 5 cc. of absolute ethyl alcohol was obtained. 1 volume of ether was then slowly added to remove all but traces of remaining pigment. Finally, the peptide, dissolved in 2 cc. of absolute ethyl alcohol, was precipitated with 20 cc. of ether; 12.5 mg. of bound PAB were present. The extract of the crude barium salt had a ratio by weight of PAB to nitrogen to carbohydrate of 1:3.34:1.03, and the purified material had a ratio of 1:1.28:0.10.

*Analytical Methods*—Nitrogen was estimated on a submicro scale by the Kjeldahl procedure with the distillation apparatus of Markham (16). Ammonia was distilled into dilute boric acid and titrated with the aid of the Scholander micro burette (17). The error of the method is about 2 per cent in a range of 20 to 100  $\gamma$ .

Amide nitrogen was estimated after aspiration from  $K_2CO_3$ , or after steam distillation, by submicro titration on samples containing about 10  $\gamma$  of ammonia. The error of these estimations is consequently about 5 per cent.

*Isolation of Products of Hydrolysis*—Hydrolysis was carried out in sealed tubes at 106° in 6 N HCl or in 5 N NaOH. L-Glutamic acid was isolated as the hydrochloride. The analytical data have been given in a preliminary publication (18). The isolation of free PAB from an acid hydrolysate and identification as the picryl derivative were also reported earlier. This has been repeated on a somewhat larger scale (11 mg.) and improved analytical data were obtained.

*Analysis*— $C_{11}H_{11}O_4N_4$ . Calculated. C 44.8, H 2.31  
Found. " 44.8, " 2.50

M.p. 284.5°, uncorrected; m.p. of an authentic sample of *p*-picrylamino-benzoic acid 284°, uncorrected; mixed m.p. 284–284.5°.

*Methods Used for Amino Acid Detection*—These methods were carried out with amounts of hydrolysate sufficient to detect the presence of at least 2 per cent of the peptide as the amino acid in question. Tyrosine and tryptophane were absent when estimation was attempted by the Lugg method (19) in an alkaline hydrolysate. Nitroprusside and cuprous mercaptide tests were negative for cystine and cysteine, as was the color reaction of McCarthy and Sullivan (20) employed to detect methionine. Serine and threonine must have been absent, for there was no increase in ammonia on treatment with periodate (21). The modified Sakaguchi reaction of Dubnoff (22) was used to investigate the presence of arginine with a negative result. The basis for the exclusion of aspartic acid has already been discussed, while lysine and histidine, though not estimated directly, have been excluded by the observation that all of the peptide nitrogen was accounted for either as amide, PAB, or  $\alpha$ -amino nitrogen and that evidence

of any basic group in the unhydrolyzed peptide, other than the arylamine of PAB (pK 2.0 (23)) is lacking. Proline has been excluded by the fact that ammonia is not formed on treatment with ninhydrin (9, 24); yet 100 per cent of the peptide nitrogen has been estimated. That leucine, isoleucine, and phenylalanine were not present was shown by the method of Virtanen *et al.* (25).

#### SUMMARY

1. A peptide of *p*-aminobenzoic acid (90 to 95 per cent pure) has been isolated from yeast in an over-all yield of about 8 per cent of the quantity of peptide estimated to be present.

2. On the basis of the nitrogen distribution and the identification of all but one component, a provisional structure has been assigned consisting of 1 terminal PAB residue, one amide group, 10 or 11 *L*-glutamic acid residues, and 1 unknown amino acid residue presumably acidic in nature.

3. The chemical and light-absorbing properties of the peptide indicate that the PAB moiety is linked to the peptide through the carboxyl group and that the arylamino group is free.

The authors are greatly indebted to Dr. Alvin F. Coburn, Dr. Randolph West, and Miss G. Corbett for help in the early stages of the problem.

*Addendum*—Some time after this manuscript was submitted for publication, the structure of folic acid was published (26). It appears that an essential part of the folic acid molecule is analogous to the PAB peptide described here. There remains to be determined, therefore, whether a functional relationship exists between the PAB peptide of yeast and the various conjugates of folic acid.

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## SPECTROPHOTOMETRIC STUDIES

### XIV. THE CRYSTALLOGRAPHIC AND OPTICAL PROPERTIES OF THE HEMOGLOBIN OF MAN IN COMPARISON WITH THOSE OF OTHER SPECIES\*

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Attention has been called in Paper XII (2) to the desirability of an ultimate standardization of the hemoglobins of those species, including man, whose blood is commonly employed in physiological and clinical investigations. With this in view the crystallization of the hemoglobins and myoglobins of several species was undertaken. At the inception of the work it was found that the procedures of Green, Cohn, and Blanchard (3) and Cannan and Redish (4), when applied to defibrinated human blood, failed to yield satisfactory crystalline products. Refractile material, presumably crystalline in nature, could be obtained, but the crystals were too small and imperfectly developed to permit crystallographic description or interpretation of form and were usually mixed with amorphous particles. Both of the above groups (3, 4) of investigators used salting-out procedures, necessitated by the unusually great solubility of human hemoglobin in reference to that of other species (3, 5). Crystallization in the laboratory of E. J. Cohn (3) was accomplished by the dialysis of hemolyzed solutions, obtained from citrated blood, against 2.8 M phosphate buffer, pH 6.8. This is a modification of a method originally developed by Theorell (6) for the crystallization of myoglobin. Cannan and Redish (4), on the other hand, added sufficient  $(\text{NH}_4)_2\text{SO}_4$  to concentrated solutions prepared from clotted cells to bring the specific gravity up to, but no higher than 1.155. The requirement of a critical concentration of  $(\text{NH}_4)_2\text{SO}_4$  was found troublesome. A slight excess of the salt beyond the necessary limit resulted in amorphous products, confirming in this respect the finding of the original authors.

In this paper a description will be given of modified techniques, which in

\* Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania. Permission for publication has been granted. A preliminary report has been presented at the December 19, 1944, meeting of the Physiological Society of Philadelphia, and an abstract has appeared (1).

many trials have consistently yielded from solutions of adult human hemoglobin beautiful, large crystals (in some cases of macro size) of the same type regardless of the salt employed in salting-out and independent of the method of anticoagulation. From this material crystallographic information was readily and successfully obtained. This permitted a crystallographic comparison of the hemoglobin of man with those of other species. These findings will be presented together with spectrophotometric data upon unusually concentrated solutions prepared from crystalline human hemoglobin. In later communications data will be given on horse and human crystalline myoglobin (1) and evidence will be furnished in support of our earlier interchangeable use of the spectrophotometric constant at  $540\text{ m}\mu$  of cyanmethemoglobin (2, 7) in studies involving the hemoglobins of dog, horse, and man, and a standard for hemoglobin (1) will be proposed.

A study of the literature suggests that the hemoglobin of man may not have been crystallized heretofore in a form suitable for reliable crystallographic characterization. Neither Green, Cohn, and Blanchard (3) nor Cannan and Redish (4) have given any information as to the crystallographic properties of their isolated products. According to Preyer (8), earlier descriptions (9-16) of crystalline human hemoglobin are unreliable. In the more recent literature the classical treatise of Reichert and Brown (17) contains reproductions of 600 photomicrographs of crystalline hemoglobin from about 200 species, but none from man. These authors state that they succeeded in one instance in preparing crystalline human hemoglobin from a placental blood clot. The oxyhemoglobin crystal class is described as *orthorhombic* and probably optically *positive*, and reduced hemoglobin as *monoclinic*. The crystals were imperfect, no interference figures were obtainable, and "no satisfactory optical characters, beyond the straight extinction [in the case of the orthorhombic form], were observed" (17). Perrier and Jannelli's finding (18) that somewhat different types of human hemoglobin crystals may be obtained from saponized blood of the new-born and adult respectively has been verified by Haurowitz *et al.* (19). The description of the crystals by the latter is neither clear nor complete. Crystals developed on a microscope slide by admixture of hemolyzed blood with saponin are for the new-born described as uniaxial and for the adult as biaxial (19). However, by dialysis against  $(\text{NH}_4)_2\text{SO}_4$  "six-sided, doubly refractive, biaxial plates" (presumably pseudohexagonal forms) were obtained from umbilical cord blood. With the same technique maternal blood yielded mainly amorphous material, mixed with a small amount of "six-sided plates or pointed needles or scalenic rhomboids, the latter biaxial" (19). It will be seen that the crystals of adult human hemoglobin obtained by us belong to the *tetragonal system*, and therefore do not correspond to any of the earlier descriptions (10, 15, 17-19).

The ultimate method of analysis of the architecture of crystals is through their effect on x-rays. This method allows the deduction of intimate details of structure, but much information of value may be revealed by classical crystallography upon which are founded the fundamental interpretations applied to Roentgen ray diffraction patterns. Except for preliminary work on rat and pig hemoglobins (20), only horse hemoglobin has been studied by the x-ray technique, but the latter substance has been investigated more thoroughly than any other crystalline protein. This work has been reviewed by Crowfoot (21) and Astbury (22), and details have emerged in reports by Perutz and his colleagues on horse methemoglobin (23, 24) and oxyhemoglobin (25), crystallized in the presence of  $(\text{NH}_4)_2\text{SO}_4$ . It is axiomatic that the theoretical *unit cell*, deduced from the characteristics of the crystal lattice, is a miniature in its dimensions, axial ratios, and optical properties of the crystal, which may be considered as an organized complex built up by an orderly, periodic repetition of the units. An example will be furnished in this paper (Table III) of the close similarity of results obtained by the x-ray (Perutz (23)) and petrographic microscope techniques (the latter used here), applied to wet and drying specimens of crystalline horse methemoglobin.

### Methods

*Crystallization Procedures*—Most of the preparations from human adult blood were made from cells recently separated by centrifugation from fresh citrated specimens, containing about 0.5 per cent of sodium citrate dihydrate (75 ml. of a 3.2 per cent solution of the salt, slightly more than isotonic, per approximately 400 ml. of blood). The essential point is the securing of solutions of hemoglobin of suitable concentration. The corpuscles are thoroughly washed and packed by successive mixing and centrifuging, one time with 0.9 per cent NaCl and three times with a mixture of 1.2 per cent saline and 0.0025 M  $\text{AlCl}_3$ . A stroma-free solution, the concentration of which in hemoglobin is of the order of 8 to 10 mM per liter (referred to a molecular weight equivalent of 16,700), is obtained from the packed cells by dilution with 1 volume of distilled water, thorough mixing with 0.4 volume of toluene, and refrigeration overnight, followed by centrifugation and siphoning off the clear hemoglobin layer. Concentration of the hemoglobin is accomplished by dialysis in a refrigerator either against saturated  $(\text{NH}_4)_2\text{SO}_4$  (700 gm. plus 1 liter of water, final volume of about 1390 ml.), the more commonly used procedure, or against 2.8 M phosphate buffer, pH 6.8 (3) (371 gm. of  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  and 160 gm. of  $\text{KH}_2\text{PO}_4$ , quantities approximated from the data of Green (26), made up to 1 liter), until the volume of the sac contents is reduced by approximately 45 per cent. Under these conditions, three-fourths to four-fifths of the sac contents

precipitates in the form of a semisolid mass of refractile granules of oxy-hemoglobin, which may be considered crystalline, but unsuitable for characterization. The dialysis is carried out in sacs made from Visking cellulose sausage casing,<sup>1</sup> with a ratio of approximately 1:3 volumes within and outside of the sac. The length of time necessary for the required concentration of the sac fluid and precipitation of three-fourths to four-fifths of the contents is, of course, a function of the relative volumes on the two sides of the membrane. The ratio given above provides for a relatively slow (6 to 24 hours) precipitation. The supernatant, concentrated solution, whose titer in hemoglobin is now approximately 14 to 17.5 mm per liter (23.4 to 29.2 gm. per 100 ml.) and whose specific gravity is of the order of 1.16, is removed from the sac. Crystallization from this solution is accomplished by the cautious addition drop by drop with thorough stirring of saturated  $(\text{NH}_4)_2\text{SO}_4$  or the 2.8 M phosphate buffer. The progress of this procedure is followed by microscopic examination, rigorous care being taken to avoid the addition of excess salt.<sup>2</sup> When definite crystallization has been initiated, the material is set aside in a refrigerator. Recrystallization may be carried out by solution and repetition of the above procedure, without change in crystal type, but, in our hands, second and third crystallizations rarely yielded as perfectly formed crystals as those originally obtained.

*Crystallization in Presence and Absence of Added Salts*—In a critical appraisal of the work of Reichert and Brown (17), whose crystallizations were usually by makeshift procedures from a drying protein ring on microscope slides and often from putrescent blood specimens, Hastings (27) has pointed out that it would be of considerable importance to reinvestigate the comparative crystallography of hemoglobins upon crystals "formed

<sup>1</sup> A product of the Visking Corporation, Chicago. A convenient size of cellulose casing, used in this work, is  $1\frac{1}{2}$  inch diameter. The simplest way to make sacs from this tubing is to seal the bottom by making a sufficiently long twist of the casing to allow for a firm tie with string, folding over, and application of a second tie. This should be done while the tubing is dry. A No. 8, 1-hole rubber stopper, grooved on the side, is tied into the upper end of the tube and two loops of thread are provided for hanging the sac on a glass rod, over the dialysis vessel. Sacs of 13 inches length, made from the  $1\frac{1}{2}$  inch casing, accommodate approximately 375 ml. of solution, while 9 inch sacs are sufficient for 250 ml. Two sacs can be subjected to effective dialysis in a 3 liter graduated cylinder, and eight sacs in a tall precipitating vessel of 15 liters capacity. The sacs may be employed over again a number of times.

<sup>2</sup> After the first appearance of typical crystals, several additional drops of  $(\text{NH}_4)_2\text{SO}_4$  (or phosphate buffer) are added. If this is not done, the crystals may redissolve on standing in the refrigerator. On the other hand, if too much salt is added, an amorphous precipitate will result. When properly crystallized, the crystals may be preserved in their mother liquor for weeks at refrigerator temperature. Crystals may also be preserved on a microscope slide, with the edges of the cover-slip sealed with Lubriscal. However, such preparations undergo distortion owing to dehydration and possibly other factors, such as pressure of the cover.

from isoelectric salt free solutions." This condition can be fulfilled with dog and horse hemoglobins which are relatively insoluble and crystallize with great ease at the pH of their isoelectric points (28), but it cannot be met in the case of human hemoglobin owing to its solubility in the isoelectric region (3). It has been suggested that the presence of salts may have some influence on crystalline form (29), or on chemical constitution (30), but the x-ray studies have been interpreted as showing that water and such salts as  $(\text{NH}_4)_2\text{SO}_4$  lie between the relatively rigid hemoglobin molecules arranged in coherent sheets, and therefore have little effect on crystalline character. However, to assure comparable conditions of crystallization for the hemoglobins of widely differing solubilities a compromise used in the present work was to study those hemoglobins (dog, horse) which crystallize spontaneously,<sup>3</sup> both in the absence and presence of added  $(\text{NH}_4)_2\text{SO}_4$  or other salts.

*Crystallography*—Commonly used terms such as needles, rods, prisms, plates, and rhombs are of little value in exact crystallographic description, particularly when they are based, as in Boor's work (31), upon photographs. The primary requisites of crystallography are a description of *crystal habit* and *classification*, determined from the elements of crystallization (the axial ratios and angles of inclination of the axes (32), and the optical character (33)). This information is obtainable by means of the petrographic microscope (32, 33). In those instances (Tables I and III) in which data on axial ratios or typical angles are given, they represent the mean of measurements upon selected, well formed crystals in the proper orientation. In the case of the tetragonal crystals of human oxyhemo-

<sup>3</sup> "Spontaneously" (in reference to crystallization) is used in a broad sense to describe the crystallization which practically always occurs with dog hemoglobin and often with that of the horse in the course of preparation, as above, of the original stroma-free solution (8 to 10 mM per liter concentration). Such spontaneous crystallization probably does not differ fundamentally from that obtained by isoelectric crystallization in methods involving the addition of acid (28). The types of crystals secured by the two procedures are identical. A number of years ago J. H. Austin and the writer found that the crystalline mesh, which formed spontaneously from concentrated hemoglobin solutions, prepared from dog and horse erythrocytes hemolyzed by freezing and thawing or by the addition of ether, could be redissolved completely by alkalization from an original pH of 6.7 to 6.9 to pH 7.6 with NaOH. In the present work alkali was not used. The crystalline material was diluted with 0.9 per cent NaCl, and the supernatant solution, approximately 1.5 to 2.0 mM per liter in hemoglobin, was separated from the undissolved crystals by centrifuging. Recrystallization of hemoglobin from the supernatant was accomplished by the addition of sufficient saturated  $(\text{NH}_4)_2\text{SO}_4$  solution to make the specific gravity 1.12. With stroma-free hemoglobin solutions I have found the following equation useful for approximating the quantity of saturated  $(\text{NH}_4)_2\text{SO}_4$  (sp. gr. = 1.239) needed to reach a desired specific gravity: ml. of saturated  $(\text{NH}_4)_2\text{SO}_4$  required = ml. of Hb solution  $\times ((\text{sp. gr. desired}) - (\text{sp. gr. of Hb solution})) / (1.239 - \text{sp. gr. of Hb solution})$ .

globin, the orientation required is the front profile (see legend to Fig. 1, A and *a* in Figs. 2 and 3). The axial ratio,  $c:a$ , where  $a = 1.0$ , is calculated

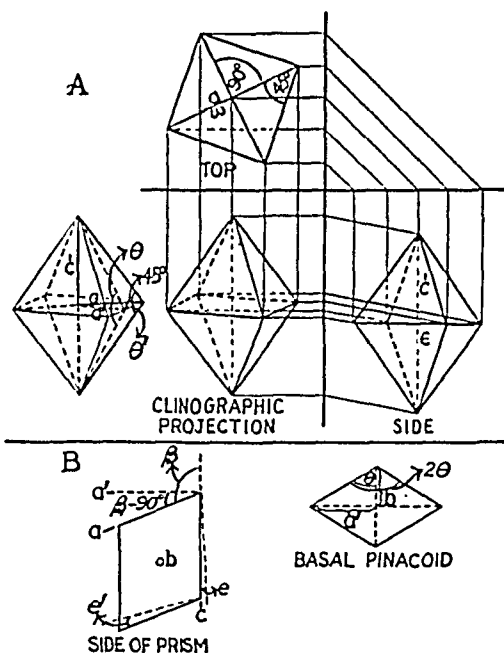


FIG. 1. A, an exact three dimensional representation of the tetragonal crystal o human oxyhemoglobin (in the presence of  $(\text{NH}_4)_2\text{SO}_4$ ), developed by means of clinographic projection from the top view and the ratio of  $c:a = 1.263:1.0$ . As shown, the top view (along the  $c$  axis) is the square base of the tetragonal bipyramid.  $c$  in the side view is taken  $= 1.263$  in comparison with  $a = 1.0$  in the top view. The angle  $\theta'$  cannot be measured with the petrographic microscope, but angle  $\theta$  can be measured in crystals in front profile orientation (as in *a*, Figs. 2 and 3). As indicated, angle  $\theta$  is bisected by  $a'$ , which is perpendicular to a side of the square base. Therefore,  $a' = a \times \sin 45^\circ$ . The dimension of  $c$  is obtained from the trigonometric relation,  $c = \tan \frac{1}{2}\theta \times a'$ . Since  $a' = a \times \sin 45^\circ$ ,  $c = \tan \frac{1}{2}\theta \times \sin 45^\circ$ . B, a representation of the monoclinic crystal of horse methemoglobin. The crystal habit developed in the monoclinic forms (Figs. 8 and 9, and Fig. 10, A and B) is the side of the prism (front profile) and the symmetrical basal pinacoid. The angle  $\beta$ , between the clino axis,  $a$ , and  $c = 111.34^\circ$ , while the dyad axis,  $b$ , is perpendicular to both  $a$  and  $c$ . The angle  $2\theta = 116.0^\circ$ .  $a'$  is drawn perpendicular to  $c$ . Hence,  $a' = a \times \cos (\beta - 90^\circ)$ . But (from the relation in the basal pinacoid),  $a' = b / \cot \theta$ ; therefore  $a = b / (\cot \theta \times \cos (\beta - 90^\circ))$ . When  $b$  is taken  $= 1.0$ ,  $a = 1 / (\cot \theta \times \cos (\beta - 90^\circ))$ .  $e$  and  $e'$  are the extinction angles, respectively, on the prism edge and on the clino edge (see Table III).

from  $c:a = \tan \frac{1}{2}\theta \times \sin 45^\circ = \tan \frac{1}{2}\theta \times 0.707$ . In the case of the monoclinic crystals of horse methemoglobin and horse oxyhemoglobin in the presence of oxalate, the orientation necessary is the front profile (side of

prism) and the basal pinacoid (Fig. 1, *B*). The axial ratio  $a:b$ , where  $b$  (the dyad axis) = 1.0, is calculated from  $a = 1/(\cot \theta \times \cos (\beta - 90^\circ))$ . (For the trigonometric relationships involved in the calculations see the legend to Fig. 1.)

*Spectrophotometry*—Concentrated solutions of all of the crystallized preparations were subjected to spectrophotometry in the Drabkin and Austin special 0.007 cm. cuvette (7); so that, in contrast to earlier preparations (3, 4, 17–19, 31), the exact character of the pigment crystallized was established.

### Results

*Tetragonal Human Oxyhemoglobin*—The crystals were a beautiful ruby-red and strongly anisotropic (birefringent) in all orientations except the square base. The crystal habit (Figs. 2 and 3) was pyramidal, with the unit bipyramids of the first order in perfect, very symmetrical development. The tetragonal nature of the crystal was proved by the optical properties and the interference figures. With many examples of orientation in the position of the isotropic square base (top view along the  $c$  axis, Fig. 1, *A*; also see Fig. 2), extinction and the typical uniaxial cross (interference figure) upon conoscopic observation were independent of the rotation of the crystal. In the front profile orientation ( $a$  in Figs. 2 and 3) extinction was straight or symmetrical (*i.e.* in positions parallel with and perpendicular to the  $c$  axis). In the position of extinction this orientation exhibited the typical uniaxial diffraction pattern on conoscopic observation. As the crystal was rotated to the bright position, the isogyres or brushes of the uniaxial cross separated, yielding a pseudobiaxial pattern. *This behavior is typical for tetragonal bipyramids.* The optical character (sign of double refraction) was *negative*; *i.e.*, the ordinary ray,  $\omega$ , proved to be the slow component and therefore had a higher index of refraction than the extraordinary ray,  $\epsilon$ , parallel to the  $c$  axis (Fig. 1, *A*). This was established without the interference figure (due to the intensity of the color of the crystals) by means of the first order red compensator plate, and was checked with the quartz wedge.

The axial ratio,  $c:a$ , where  $a = 1.0$ , was determined accurately upon a sufficiently large number of crystals (Table I) for the main crystallizations from citrated specimens in the presence of  $(\text{NH}_4)_2\text{SO}_4$  or phosphate. Apparently the crystals obtained with sulfate or phosphate are practically identical (Figs. 2 and 3; Table I). The use of citrated or oxalated blood definitely aided crystallization, but similar crystals were obtained from defibrinated specimens (in the absence of anticoagulants; Table I).

Twinning, mainly from the faces of the prism (interpenetrant twins, *t*, Figs. 2 and 3) occurred very early in the crystallization and increased

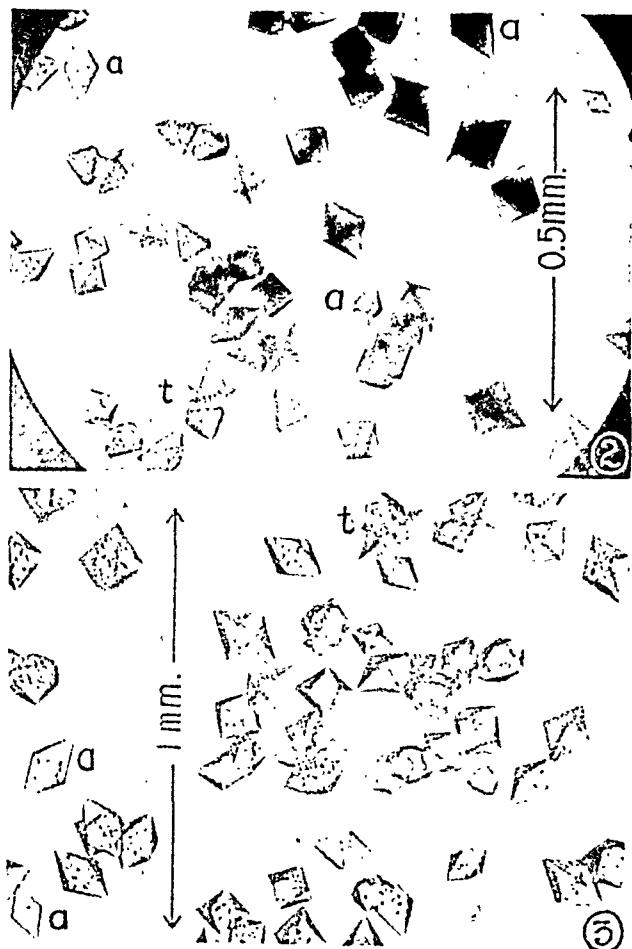


FIG. 2. Tetragonal human oxyhemoglobin crystallized (from stroma-free concentrated solutions prepared from originally citrated blood specimens) in the presence of  $(\text{NH}_4)_2\text{SO}_4$ .

FIG. 3. Tetragonal human oxyhemoglobin crystallized (from stroma-free concentrated solutions prepared from originally citrated blood specimens) in the presence of 2.8 M phosphate buffer, pH 6.8. In each case the perfectly symmetrical unit bipyramids are shown in random orientation. Front profile orientations (*a*) were used for measurement of the apical angle and angle  $\theta$ . Imperfect examples of the square base orientations are present mainly in Fig. 2 (compare with "top view" in Fig. 1, A). Typical examples of interpenetrant twins are designated by *t*. The linear dimensions are based on the determined magnification factors.

progressively with crystal growth in bulk. Secondary growth on non-twinned crystals also occurred. This factor as well as pressure and possibly dehydration produced distortion in the crystals. The optical character



and the tetragonal nature (typical interference figure, etc.) were preserved, but the axial angles and ratios (difficult to measure with the petrographic microscope in these older large crystals) were markedly altered, as is illustrated in Table I. For conditions of optimal measurement freshly crystallized specimens were employed. Pressure on the cover-slip was avoided, since the crystals were soft and easily fractured.

TABLE I

*Axial Angles and Axial Ratio,  $c:a$ , of Crystalline Adult Human Oxyhemoglobin\**

Nature of specimen	Salt used	No. of crystals measured	Angle $\theta$ † (mean values)	Apical angle‡ (mean values)	Axial ratio, $c:a$ ‡
			<i>Degrees</i>	<i>Degrees</i>	
Citrated, fresh.....	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	30	121.53 $\pm$ 0.48	59.63	1.263
“ old growth§..	“	6	135.0 $\pm$ 1.0	46.4	1.706
“ fresh.....	Phosphate buffer	26	123.87 $\pm$ 0.40	59.2	1.326
Oxalated, “¶.....	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	18	121.55 $\pm$ 0.24	59.63	1.265
Debrinated, fresh.....	“	5	121.8 $\pm$ 0.5	59.6	1.271
“ “ .....	Phosphate buffer	8	123.75 $\pm$ 0.12	59.4	1.324

\* Character of pigment derivative established spectrophotometrically (see the text and Table II).

† See Fig. 1,  $A$  and  $a$  in Figs. 2 and 3.

‡ Axis  $a = 1.0$ ;  $c = \tan \frac{1}{2}\theta \times \sin 45^\circ$ .

§ Growth in bulk at refrigerator temperature in beakers covered with watch-glasses for a period of 7 to 10 days. Under these conditions a tendency towards conversion to ferrihemoglobin (methemoglobin) was demonstrated spectrophotometrically. In 10 days the amount of oxidation (formation of methemoglobin) is of the order of 1 to 3 per cent (see foot-note 5).

|| Owing to the nature of the secondary growth the measurement of these angles was uncertain (see the text).

¶ Similar crystals were obtained in the presence of 1 per cent oxalate, added to stroma-free solutions prepared from originally defibrinated blood specimens.

The spectrophotometric data upon solutions of freshly crystallized adult human hemoglobin collected in Table II (Experiments 2 to 5) establish unequivocally that no detectable alteration in the character of the pigment from that present in normal, hemolyzed blood, exposed to air (Experiment 1), occurred in crystallization. On the basis of the excellent agreement of the absorption constants ( $\epsilon$  values, defined in the legend to Table II) for HbO<sub>2</sub> in the different preparations (Column 7, Table II), the unusually high ratios of  $\epsilon_{573}:\epsilon_{562}$  and  $\epsilon_{541}:\epsilon_{562}$  (Columns 8 and 9), and the closely agreeing  $\epsilon$  values at the 555 m $\mu$  maximum for Hb (Column 11), prepared from corresponding HbO<sub>2</sub> solutions by means of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, the conclusion

TABLE II

Comparison of Extinction Coefficients,  $\epsilon$ , for Oxygenated Hemoglobin and Deoxygenated Hemoglobin, on Original Adult Human Blood Specimens and on Solutions from Crystallized Adult Human Hemoglobin Preparations

Experiment No.	Nature of preparation	No. of specimens	Total pigment* (4)	Cuvette depth (5)	HbO <sub>2</sub>				Hb	
					Wave-length (6)	$\epsilon$ (7)	Extinction ratios		Wave-length (10)	$\epsilon$ (11)
							$\epsilon_{578}:\epsilon_{562}$ (8)	$\epsilon_{578}:\epsilon_{542}$ (9)		
(1)					m $\mu$				m $\mu$	
1	Whole blood, hemolyzed	6	7.76-8.63	0.007†	578§	15.34 $\pm$ 0.08	1.81	1.72	555§	13.55 $\pm$ 0.06
2	"Crystalline" ppt. in dialysis sac	1	0.0770	1.0	562¶	8.50 $\pm$ 0.06				
					542§	14.61 $\pm$ 0.09	1.83	1.73	555§	13.65
3	Tetragonal HbO <sub>2</sub> (in presence of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> )	1	32.8	0.007†	562¶	8.40				
					542§	14.53	1.81	1.73		
					578§	15.35				
3a	501-fold dilution of HbO <sub>2</sub> in Experiment 3 with H <sub>2</sub> O	1	0.0654	1.0	562¶	8.46				
					542§	14.65	1.80	1.73	555§	13.49
4	Tetragonal HbO <sub>2</sub> (in presence of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> )	1	38.2	0.007†	578§	15.35				
					542§	8.50	1.82	1.74		
					578§	14.76				
4a	501-fold dilution of HbO <sub>2</sub> in Experiment 4 with H <sub>2</sub> O	1	0.0762	1.0	562¶	15.41				
					542§	8.48	1.82	1.74	555§	13.57
					578§	14.79				
5	Salt-free dialysate of crystalline HbO <sub>2</sub>	1	0.1083	1.0	562¶	15.17				
					542§	8.33	1.80	1.72	555§	13.44
					578§	14.47				
					562¶	15.43				
					542§	8.58				
					542§	14.77				

As previously employed, our  $\epsilon$  values may be defined as *fractional molecular extinction coefficients* (34) and are for a concentration of 1 mm per liter (in the case of hemoglobin, referred to an equivalent weight of 16,700) and a depth of 1 cm. Thus,  $\epsilon = (1/(c \times d)) \times \log I_0/I$ , where the concentration  $c$  is expressed in mm per liter, the depth  $d$  in cm., the original intensity  $I_0$  is 1.0, and the intensity of transmitted light  $I$  is expressed as a fraction of unity. The spectral interval (band width) employed was 1.5 to 2.0 m $\mu$ .

Experiment 1, blood hemolyzed by means of saponin; specimens exposed to air (2).

Experiment 2, dialysis of stroma-free hemoglobin solution, prepared from an originally citrated blood specimen, against saturated  $(\text{NH}_4)_2\text{SO}_4$ ; the "solid" contents of the sac (the material analyzed) were by weight 35.4 per cent  $\text{HbO}_2$  and 64.6 per cent water and salts.

Experiments 3 and 4, solutions of tetragonal oxyhemoglobin (Fig. 2) crystallized from the concentrated supernatant solutions, obtained from the dialysis sacs (see "Methods"). The original blood samples were citrated. The pH of these solutions, determined by the glass electrode on diluted specimens, Experiments 3a and 4a, was 5.93.

Experiment 5, preparation, crystallized in the presence of  $(\text{NH}_4)_2\text{SO}_4$ , dialyzed in the refrigerator against water (double distilled in glass) until free of chloride and sulfate. The original concentration of the dialysate in hemoglobin was 5.16 mm per liter. Similar results were obtained by dialyzing against water until salt-free the "crystalline" precipitate in the dialysis sac after the original dialysis against  $(\text{NH}_4)_2\text{SO}_4$  (see Experiment 2).

\* Total pigment determined spectrophotometrically upon aliquots converted to cyanmethemoglobin,  $\text{MtlbCN}$ , with  $\epsilon = 11.5$  at 540 m $\mu$  as a constant for the latter (2, 35). To obtain gm. per 100 ml. multiply the values by factor 1.07.

†  $\text{Hb}$  obtained from  $\text{HbO}_2$  by addition of solid  $\text{Na}_2\text{S}_2\text{O}_4$  (0.1 mg. per ml.).

‡ Drabkin and Austin special cuvette (7).

§ Location of maxima of absorption.

|| The mean for the six specimens, with the standard error,  $= \pm \sqrt{2\bar{d}^2/n(n-1)}$ .

¶ Location of the minimum between the two maxima of  $\text{HbO}_2$  in the green spectral region.

must be reached that the crystallized preparations were of unchanged oxyhemoglobin. Limitations in technique do not permit a decision as to the possible presence of similar quantities of ferrihemoglobin (methemoglobin) of an order of less than 0.5 per cent of the total pigment in both the original blood and the crystalline specimens. But the present data fortify our earlier conclusion (2) that the presence of more than this minimal quantity of MIIb appears to be incompatible with the findings.

Particular attention may be directed to Experiments 3 and 4, Table II, which attest to the unusual solubility of human oxyhemoglobin (3). In spite of the presence of a high concentration of  $(\text{NH}_4)_2\text{SO}_4$ , at a pH of 5.93 (determined by the glass electrode), solutions were measured spectrophotometrically in the Drabkin and Austin special cuvette (7) at a concentration in oxyhemoglobin of 32.8 and 38.2 mm per liter (54.8 and 63.8 gm. per 100 ml., respectively). The agreement of the  $\epsilon$  values obtained on these very concentrated solutions with corresponding values from measurements upon 501-fold diluted samples in cuvettes 1 cm. deep (Experiments 3a and 4a) provides evidence that the pigment was in true solution. The concentration of 63.8 gm. per 100 ml. is double that normally present in the erythrocytes of man and is believed to be the highest concentration of hemoglobin yet studied by optical or other physical techniques. Drabkin and Austin (7) some years ago measured horse oxyhemoglobin spectrophotometrically at a concentration of 42.7 gm. per 100 ml., but in the absence of salts and at pH 7.8. The concentration of 63.8 gm. per 100 ml., attained in the present work, represents the limit at which solutions of hemoglobin can be measured with any degree of accuracy in the 0.007 cm. cuvette. Such unusually concentrated solutions were found to gel on standing exposed to air for a short period of time.

*Preparation of Solutions for Standardization, and Preservation of Hemoglobin*—Experiment 2, Table II, is an example of data obtained from solutions of the refractile, crystalline-like precipitate formed in the sacs upon dialysis against saturated  $(\text{NH}_4)_2\text{SO}_4$ . Although this material was unsuitable for crystallographic characterization, it was in the fresh state unaltered oxyhemoglobin, and was used to provide salt-free solutions for studies on hemoglobin standardization (1) to be reported later. The salt-free solutions were prepared either from this precipitate or from the tetragonal crystals, by dialyzing their solutions at refrigerator temperature against water (double distilled in glass to avoid metallic contamination) until the dialysate was free of chloride and sulfate. (My colleague, Dr. J. H. Jones, has reported to me that such hemoglobin preparations are also practically free of phosphate.)

The measurements recorded in Experiment 5, Table II, indicate that the procedures of crystallization and dialysis, which required about 1 week,

produced no change in the hemoglobin. For preservation the salt-free oxyhemoglobin solution was saturated with pure carbon monoxide, and either stored as such in the refrigerator or in vacuum-sealed bottles in the dry form, following desiccation in the frozen state. At refrigerator temperature the HbCO solutions were found to remain unchanged for several months, while the preparations vacuum-dried in the frozen state (of which several hundred gm. were made) appeared to be extraordinarily stable. Except for a very slow conversion to MHb, such preparations may be preserved for years. Since 1937, when the writer and E. W. Flosdorf (36) first prepared hemoglobin derivatives by the "lyophilization" process, occasional periodic tests have been made upon the preparations, stored in sealed containers in a refrigerator. Specimens of lyophilized dog cyanmethemoglobin have remained unaltered for 9 years. Preparations of hemoglobin (tested for a period of 5 years) were partially oxidized to MHb,<sup>4</sup> but were undenatured in the sense that total pigment still remained completely convertible to Hb by means of  $\text{Na}_2\text{S}_2\text{O}_4$ .

*Orthorhombic Dog and Horse HbO<sub>2</sub>*—Figs. 4 and 5 (for dog) and Figs. 6 and 7 (for horse) illustrate the crystal habit of bulk crystallizations of dog and horse HbO<sub>2</sub>. Figs. 4 and 6 are specimens of spontaneous crystallizations<sup>3</sup> from concentrated stroma-free solutions prepared from defibrinated blood, while Figs. 5 and 7 are corresponding recrystallizations from dilute solutions in the presence of  $(\text{NH}_4)_2\text{SO}_4$  to afford conditions approaching those used for the crystallization of the very soluble human HbO<sub>2</sub>. It is obvious that this salt had little effect on the character of the crystals. The crystals in all cases were long prisms, often of capillary form. The orientations of these crystals prepared in bulk are such that axial angles could not be measured. However, the extinction was straight (parallel with and perpendicular to the brachypinacoid or *c* axis), and in larger crystals the

<sup>4</sup> Results obtained by the writer are in full agreement with the recent report of Farr and Hiller (37) that dry hemoglobin with only minimal quantities of MHb can be prepared by vacuum drying from the frozen state, and that such preparations when exposed in the dry state to air are extraordinarily susceptible to conversion to MHb. It may be stated that qualitatively this change towards ferrihemoglobin is not unusual. The change occurs slowly and progressively when dilute solutions of oxyhemoglobin are allowed to stand exposed to air (38), and more rapidly when material such as the solid precipitate obtained in the dialysis against saturated  $(\text{NH}_4)_2\text{SO}_4$  is stored in non-evacuated containers. Here the conversion can be followed by the progressive change in color from the surface downwards. Tetragonal crystals of HbO<sub>2</sub> undergoing slow dehydration on a microscope slide have also been found to be partially converted to MHb. Such conversion in itself has apparently little influence on the crystal system, and suggests that human HbO<sub>2</sub> and MHb (formed after crystallization) may be crystallographic pseudomorphs. It does not follow, however, that human MHb formed before crystallization need crystallize in the tetragonal system (see the case of horse MHb in the text).

biaxial interference pattern was observed. The crystal system is therefore orthorhombic. No monoclinic forms (see below) were observed in such

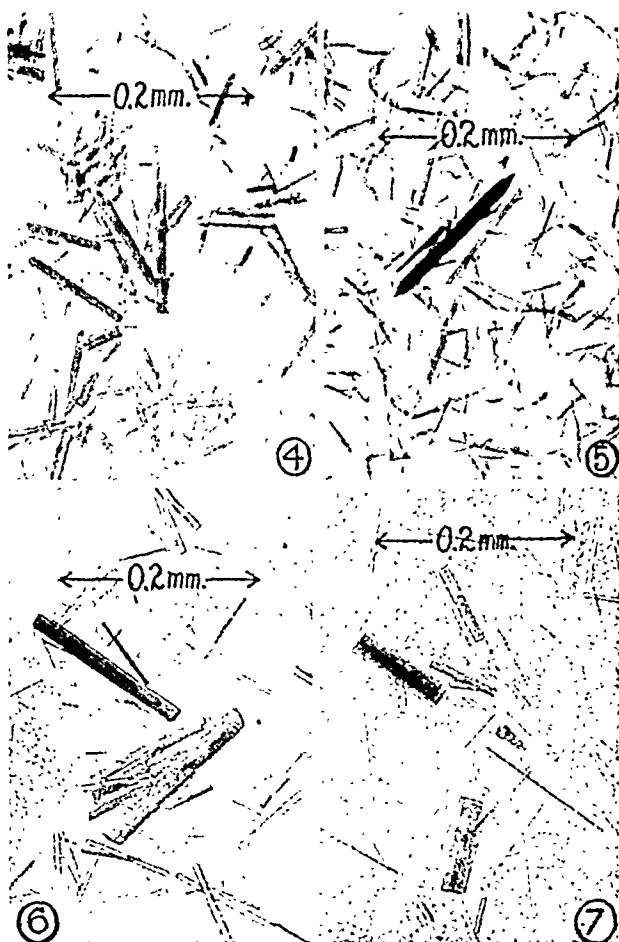


FIG. 4. Orthorhombic crystals of dog oxyhemoglobin crystallized spontaneously from stroma-free solutions prepared from defibrinated blood specimens.

FIG. 5. The same recrystallized in the presence of  $(\text{NH}_4)_2\text{SO}_4$ . The preparation shown in Fig. 4 was used, and solution followed by recrystallization was by the procedure described in foot-note 3.

FIG. 6. Orthorhombic crystals of horse oxyhemoglobin crystallized spontaneously from stroma-free solutions prepared from defibrinated blood specimens.

FIG. 7. The same recrystallized in the presence of  $(\text{NH}_4)_2\text{SO}_4$ . The preparation shown in Fig. 6 was dissolved and recrystallized according to the procedure in foot-note 3. The linear dimensions are based on the determined magnification factors.

crystallizations. It was established spectrophotometrically that the crystallized specimens were oxyhemoglobin. With these less soluble crystals of dog and horse  $\text{HbO}_2$  it was possible to convert them completely to MHb

by means of a drop of concentrated  $K_3Fe(CN)_6$  added to the specimens on a microscope slide. Conversion was accomplished without evident change in

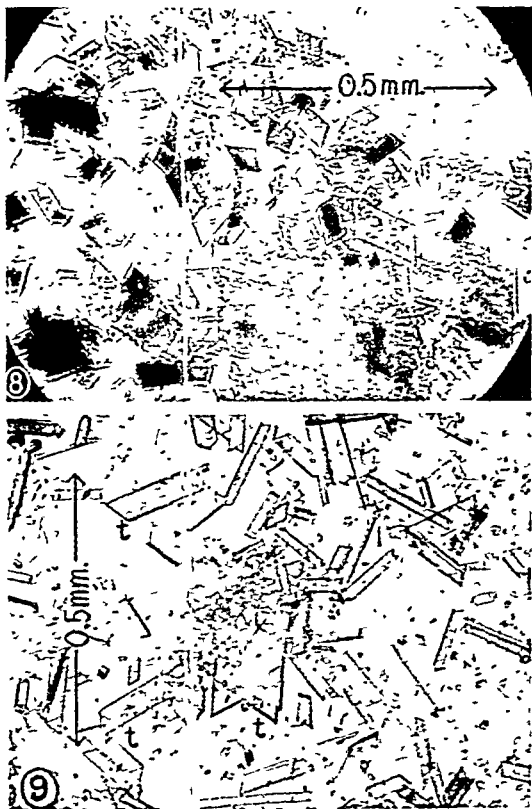


FIG. 8. Monoclinic crystals of horse oxyhemoglobin crystallized from stroma-free solutions by acidification to pH 6.6 in the presence of 3 per cent potassium oxalate. (In the absence of oxalate typical orthorhombic crystals, as in Fig. 6, were obtained from an aliquot of the same hemoglobin solution.)

FIG. 9. Monoclinic crystals of horse oxyhemoglobin recrystallized in the presence of 3 per cent potassium oxalate and  $(NH_4)_2SO_4$ . The preparation shown in Fig. 8 was dissolved by acidification to pH 6.0 and recrystallized by addition of  $(NH_4)_2SO_4$ . Twins typical of horse hemoglobin are indicated by *t*. The linear dimensions are based on the determined magnification factors.

crystal type. This is a good example of pseudomorphism for crystals of dog and horse  $HbO_2$  and MHb.

*Monoclinic Horse  $HbO_2$  in Presence of Oxalate*—In confirmation of Reichert and Brown (17), monoclinic horse  $HbO_2$  crystals (Figs. 8 and 9

and Experiment 1, Table III) were secured either from stroma-free solutions prepared from blood to which potassium oxalate had been added (final concentration of salt, 3 per cent) or from stroma-free solutions to which oxalate in the above concentration was added. It is clear from the photo-

TABLE III

*Axial Angles and Axial Ratios of Monoclinic Horse Oxyhemoglobin\* and Methemoglobin† and Effect of Drying on Crystalline Character of Horse Methemoglobin*

Experiment No.	Character of preparation	Angle $\beta$ (Fig. 1, B)‡	Angle $2\theta$ (Fig. 1, B)‡	Extinction angles (Fig. 1, B)	Axial ratios $a:b$ §
		degrees	degrees	degrees	
1	HbO <sub>2</sub> , in presence of 3% oxalate and (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (Fig. 9)	102.5 $\pm$ 0.6	114.0 $\pm$ 0.5	4.5   9.8¶	1.577
2	MHb, in presence of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> **	111.34 $\pm$ 0.20	116.0 $\pm$ 0.1	4.0   6.5-9.9¶	1.720 (1.708)††
3a††	MHb, in presence of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	111.3 (111.1)††	116.0		1.718 (1.708)††
3b††		114.7	114.6		1.712
3c††		118.0 (116.2)††	113.2		1.718 (1.708)††
3d††		122.3 (127.5)†† (137.5)††	110.2		1.695 (1.708)†† (1.630)††

\* Established spectrophotometrically.

† Conversion to MHb before crystallization by addition of 1.1 moles of K<sub>3</sub>Fe(CN)<sub>6</sub> per 1 mole of hemoglobin iron.

‡ Mean values, except in Experiment 3, in which individual crystals were measured.

§ Axis  $b = 1.0$ ;  $a = 1/(\cot \theta \times \cos (\beta - 90^\circ))$ .

|| Extinction angle on prism edge ( $e$ , Fig. 1, B).

¶ Extinction angle on clinopinacoid ( $e'$ , Fig. 1, B).

\*\* Practically identical crystals are obtained in absence of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Fig. 10).

†† The values in parentheses are based on x-ray measurements, under comparable conditions, by Perutz (23).

‡‡ Measurements on the same crystals (one in the side of the prism and the other in the basal pinacoid orientation (Fig. 1, B)) during progressive slow drying (over a period of 7 days) on a microscope slide.

micrographs (Figs. 8 and 9) that the bulk of the crystallization is in the monoclinic system. The axial angles, including the characteristic oblique extinction angles, and axial ratios for these crystals are given in Experiment 1, Table III. One brush of the biaxial interference figure, eccentric to the field and revolving as the crystals were revolved on the stage, was secured on conoscopic observation from the basal pinacoid orientations



(Fig. 1, B) This behavior suggests that these crystals were optically *positve* (17). A comparison of Figs. 8 and 9 reveals that, as in the case of the orthorhombic crystals of dog and horse HbO<sub>2</sub>, the presence of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> did not alter the crystalline character of the monoclinic crystals of horse HbO<sub>2</sub>.

*Monoclinic Crystals of Horse MHb*—Either in the pre-ence or absence of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, the typical habit of crystals made from stroma-free solutions



[FIG 10 Monoclinic crystals of horse ferrihemoglobin (methemoglobin) crystallized spontaneously from stroma free solutions to which  $K_3Fe(CN)_6$  had been added in a concentration of 1.1 moles of ferricyanide to 1 mole of hemoglobin iron 1 and B are identical photomicrographic fields with the Nicol prisms uncrossed and crossed respectively Crystals oriented at the proper oblique angle in respect to the axes (indicated by crossed arrows *H* and *V*) to show complete extinction are designated by *a* and *b*, which correspond in A and B The linear dimension is based on the determined magnification factor

of horse MHb (conversion to MHb before crystallization by means of 1.1 moles of  $K_3Fe(CN)_6$  per 1.0 mole of hemoglobin iron) was monoclinic (Fig. 10 and Experiment 2, Table III) When horse hemoglobin was in the oxidized form prior to crystallization, in contrast to conversion after crystallization, no orthorhombic crystals were found in the specimens

The orientations most frequently observed in the thin plate-like monoclinic forms were the side of prism and symmetrical basal pinacoid (Fig. 1, B). Measurements upon these sections yielded the data summarized in Table III. Periodic measurements upon the same individual crystals, respectively in the side of prism and basal pinacoid orientations, during

progressive slow evaporation of the mother liquor on the microscope slide, revealed the distortion produced as a consequence of dehydration (Experiments 3a to 3d, Table III). The data of Perutz (23) obtained by x-ray analyses of slowly drying horse MIIb in the presence of  $(\text{NH}_4)_2\text{SO}_4$  are included for comparison. It appears that in this connection similar results are yielded by the petrographic microscope and x-ray techniques.

#### DISCUSSION

In this study of the crystallization under similar conditions of hemoglobin in bulk of three species, man, dog, and horse, three distinct crystal systems have been revealed: tetragonal, orthorhombic, and monoclinic. Reichert and Brown (17) assumed that horse HbO<sub>2</sub>, for example, is dimorphic, crystallizing in both the orthorhombic and monoclinic systems. The same systems have been found in the present work, but monoclinic forms of HbO<sub>2</sub> were not observed in the absence of oxalate. Hence, for the present the question of such dimorphism must remain unsettled, since the crystallization in the monoclinic system may be due to an actual combination of the hemoglobin with oxalate (29, 30). However, I have demonstrated that high concentrations of such salts as  $(\text{NH}_4)_2\text{SO}_4$  are without effect on the crystal class, thereby allowing a direct comparison of the crystalline forms in hemoglobins of widely differing solubilities.

Reichert and Brown (17) further believed that all the crystalline hemoglobins are members of an isomorphic or isopolymorphic series. In support of this hypothesis a definite relationship appeared to exist in the axial angles of the biaxial crystals (orthorhombic and monoclinic forms). The uniaxial (tetragonal and hexagonal forms) could not be fitted into this scheme. Tetragonal-like crystals were observed by Reichert and Brown only in the hemoglobins of the fallow deer and the collared peccary, but these crystals were so small that interference figures could not be obtained. Reichert and Brown (17) therefore concluded that such forms could represent mimetic twins of the orthorhombic system. The hemoglobin of man, prepared by the writer, thus provides an exception to Reichert and Brown's rule. Crystalline human hemoglobin may be unique, but it is definitely a true tetragonal form, and hence the value and applicability of the theory of isomorphism of hemoglobins may be questioned.

I am highly indebted to Dr. A. W. Postel, Department of Geology, University of Pennsylvania, for checking my conclusions with reference to the crystallographic character of human hemoglobin, and for his helpful advice. Acknowledgment is due also to Dr. D. H. Black of the University Hospital for supplying periodically large quantities of human cells, collected from blood drawn from normal subjects for the blood bank, and to

Miss H. Lorraine Leidy for technical assistance. The excellent photomicrographs in Figs. 2 to 9 were made by Mr. Basil B. Varian of the Department of Anatomy.

#### SUMMARY

The preparation of concentrated stroma-free solutions of hemoglobin has been described. From such solutions the hemoglobin of adult man has been successfully crystallized in a form suitable for crystallographic characterization. The crystalline products have been identified spectrophotometrically as unaltered oxyhemoglobin.

Owing to the great solubility of human hemoglobin, its crystallization was accomplished in the presence of  $(\text{NH}_4)_2\text{SO}_4$  or phosphate buffer. In each case similar, relatively large crystals were obtained in the habit of perfectly symmetrical bipyramids. The character of the crystals has been shown to be independent also of the method of anticoagulation employed in the original blood specimens. The crystal system was proved to be tetragonal, which is unique for crystalline hemoglobins. Crystallographic information was secured by means of the petrographic microscope and has been presented.

The less soluble hemoglobins of the dog and horse have been crystallized both in the absence and presence of  $(\text{NH}_4)_2\text{SO}_4$ . High concentrations of the latter salt were found not to affect the crystalline character. The crystallization of dog and horse  $\text{HbO}_2$  in the presence of  $(\text{NH}_4)_2\text{SO}_4$  afforded for the first time the possibility of comparing the hemoglobins of the three species, man, dog, and horse, when crystallized in bulk under comparable conditions. In these three species three distinct crystal systems were found: tetragonal, orthorhombic, and monoclinic. The validity of Reichert and Brown's hypothesis (17) that all the crystalline hemoglobins belong to an isomorphic series has been questioned, largely owing to the discovery of the tetragonal (uniaxial) human hemoglobin.

From the crystallized preparations of man, dog, and horse, salt-free solutions of unchanged oxyhemoglobin have been prepared by dialysis against water. Such solutions after conversion of the pigment to carbonyl hemoglobin ( $\text{HbCO}$ ) may be preserved unchanged for several months in the refrigerator, or the pigment may be vacuum-dried in the frozen state. In vacuum-sealed containers at refrigerator temperature such preparations have remained undenatured for years.

Owing to the great solubility of crystallized human  $\text{HbO}_2$ , it was possible to obtain accurate spectrophotometric data upon unusually concentrated solutions of this pigment. One such solution had a concentration in the presence of  $(\text{NH}_4)_2\text{SO}_4$  of 38.2 mM per liter or 63.8 gm. per 100 ml., approximately double the concentration of hemoglobin in the normal erythrocytes

of man, and probably the highest concentration of hemoglobin yet studied by optical means.

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# A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF PENICILLIN\*

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A physicochemical method has been developed by which 5 to 75  $\gamma$  (8 to 120 Oxford units) of penicillin per ml. may be determined within an error of  $\pm 5$  per cent. The procedure requires about 20 minutes for one complete analysis, but a large number of samples may be analyzed at one time. Since crystalline penicillins G, X, K, and F have nearly the same extinction per unit weight, a single measurement of any preparation containing these penicillins in any proportion determines the total penicillin present.

## Principle

Crystalline salts of pure penicillins G, X, K, and F, freshly dissolved in acetate buffer, pH 4.6, do not absorb in the region of 290 to 360  $m\mu$  (see Fig. 1 and Table II). After heating in this buffer they absorb strongly in this region, with a maximum at 322  $m\mu$ . The impurities in crude preparations absorb in this region but the increase on heating is due only to penicillin (see Fig. 3). The present method consists of measurement of the change in absorption at 322  $m\mu$  produced by heating under controlled conditions and comparing this change to that of a similarly treated standard preparation.

The preparations used are listed in Table I.

## Procedure

Two related procedures have been used: the first one, Procedure A, when ample quantities of more concentrated solutions were available; the second, Procedure B, when the concentration or quantity was low.

*Procedure A*—A 2 ml. aliquot of a penicillin solution containing 20 to 300  $\gamma$  per ml., or roughly 35 to 500 Oxford units per ml., was added to 6 ml. of 0.4 M acetate buffer,<sup>1</sup> pH 4.6, in a 30 to 50 ml. test-tube. 4 ml. of this solution were pipetted into another test-tube. One of the tubes was placed in boiling water for 15 minutes, after which it was cooled rapidly to room temperature. The absorption density of the heated and unheated aliquots was determined at 322  $m\mu$  in a Beckman D.U. quartz photoelectric spectrophotometer (1).

\* This work was done in consultation with the Committee on Medical Research of the Office of Scientific Research and Development.

<sup>1</sup> The ratio of molar concentrations of sodium acetate to acetic acid is 1.

*Procedure B*—To 4 ml. of a penicillin solution containing 5 to 50  $\gamma$  per ml., or 8 to 80 Oxford units per ml., was added 0.25 ml. of a 5 M acetate buffer solution, pH 4.6.<sup>1</sup> The final concentration and pH were close to that in Procedure A. The absorption density of this solution was determined before heating and then again after heating, as in Procedure A.

*Notes*—(a) The micrograms per ml. in the above procedures refer to pure penicillin. (b) The  $E_{1\text{ cm}}^{1\%}$  used later in this paper is equal to  $E_{1\text{ cm}}^{1\%} \times 10^{-4}$  and is the absorption density per microgram per cm. of depth or

TABLE I  
Materials

Preparation	Bioassay; <i>Staphylococcus aureus</i> units per mg.	Elementary analyses,* per cent					
		C	H	N	S	Na	H <sub>2</sub> O
Crystalline sodium penicillin G†(CrA-152-60)		53.94 53.75	4.77 4.87			6.45 6.36	
Crystalline ammonium penicillin K† (AV-73)	1750–2000‡	53.46 53.22	8.13 8.09	11.69 11.62	8.99 8.92		3.5
Crystalline sodium penicillin X§							
Crystalline penicillin F   (175E-ANW-6)	1632	50.27 50.19	5.73 5.94	8.38 8.56			
Commercial, Penicillin 1006¶	368						
“ “ 999¶	1000						

\* The upper reading for each preparation is the calculated value; the lower reading, found.

† Supplied by Dr. O. Wintersteiner, The Squibb Institute for Medical Research, New Brunswick, New Jersey.

‡ The *Bacillus subtilis*-*Staphylococcus* ratio = 0.3 to 0.4.

§ Supplied by Dr. Henry Welch, Food and Drug Administration, Washington, D. C.

|| Supplied by Dr. G. F. Cartland and Dr. A. N. Wick, The Upjohn Company, Kalamazoo, Michigan.

¶ Supplied by E. R. Squibb and Sons, New Brunswick, New Jersey.

thickness. (c) In Procedure A it was found convenient to analyze the unheated sample while the other aliquot was being heated. (d) During the time of heating a small funnel in the mouth of the test-tube served as a reflux condenser. (e) Reproducible conditions must be maintained, particularly when a standard penicillin is not analyzed simultaneously. A constant volume of boiling water, the level of which was kept just above the level of the sample in the test-tube, has been satisfactory. Depending on the number of samples heated at one time, the source of heat and volume of water should be such that introduction of the tubes causes the temperature of the water to drop no more than 2–3°.



*Conversion of Observed Increase in Absorption Density to Penicillin Concentration*

There are several related procedures for accomplishing this conversion which are worth noting for their usefulness under different circumstances. As will be discussed in another section, the change in absorption does not increase exactly linearly with increasing concentrations of penicillin; *i.e.* the specific extinction,  $E_1^1 \gamma_{cm}$ , is not constant (see Table III).<sup>2</sup> Were it not for this slight deviation, conversion would be simple.

The three following procedures may be used.

Empirical curves, such as are shown in Figs. 2, *A* and 2, *B*, have resulted from plotting the averages of the values in Table III. It is important to have a scale large enough to permit a precise conversion, particularly in the region of low concentration (see Fig. 2, *B*).

A less precise, but convenient, procedure assumes no deviation from linearity; *i.e.*, Beer's law is obeyed. The observed increase in density is divided by the  $E_1^1 \gamma_{cm} = 0.022$ . All results obtained by this method will be correct within  $\pm 10$  per cent.

Another procedure, which has certain obvious advantages, particularly if the conditions of the procedure have to be altered to any degree, is the comparison of the density of the unknown solutions with those of simultaneously analyzed standards.

*Results*

*Different Kinds of Penicillin*—Table II contains the absorption densities at various wave-lengths of 25  $\gamma$  per ml. of crystalline sodium penicillins, G, X, K, and F, before heating, as well as after heating. It may be seen that the different penicillins show almost identical absorption densities at any wave-length between 290 and 400  $m\mu$ . There is practically no absorption over this range before heating, but after heating the absorption is very strong, with a maximum at 322  $m\mu$ . This is shown graphically in Fig. 1 where, to eliminate confusion, only the curves of crystalline sodium penicillin G have been plotted.

The results shown in Table II indicate that the spectrophotometric analyses are independent, within the limits already stated, of the nature or proportion of the various penicillins. To determine the extent of any possible interaction or influence of one penicillin on another, particularly during heating, equal quantities of the four kinds of penicillins were dissolved together and analyzed at different levels of concentration. In every instance the spectrophotometric analysis of this mixture agreed within 5 per cent of the correct total concentration of penicillin.

<sup>2</sup> This inconstancy of  $E_1^1 \gamma_{cm}$  was brought to the writer's attention by Dr. Frank H. Wiley of the Food and Drug Administration, Washington, D. C.

*Effect of Penicillin Concentration on Absorption Density*—The analyses of various concentrations of crystalline sodium penicillin G are shown in Table III and Fig. 2. It may be seen that the  $E'_{\text{cm}}^1$  increases from about

TABLE II  
*Ultraviolet Absorption Spectra*

25  $\gamma$  per ml. of crystalline sodium penicillins G, X, K,\* and F, before and after heating 15 minutes at 100° in 0.3 M acetate buffer, pH 4.6.

Wave-length  m $\mu$	Absorption densities, log $\frac{I_0}{I}$							
	Before heating				After heating			
	Penicillin				Penicillin			
	G	X	K	F	G	X	K	F
400	0.010	0.000	0.003	0.005	0.001	0.000	0.000	0.002
375	0.000	0.000	0.000	0.003	0.006	0.006	0.006	0.010
360	0.000		0.001	0.003	0.027		0.030	0.033
350	0.002	0.000	0.002	0.002	0.115	0.120	0.120	0.120
345	0.004	0.000	0.005	0.001	0.218	0.220	0.223	0.220
340	0.005	0.000	0.009	0.002	0.345	0.340	0.343	0.343
335	0.007	0.002	0.010	0.001	0.445	0.440	0.455	0.450
330	0.009	0.002	0.012	0.002	0.520	0.505	0.535	0.530
325	0.010	0.003	0.015	0.004	0.570	0.550	0.595	0.580
322	0.010	0.003	0.015	0.005	0.580	0.570	0.610	0.590
320	0.010	0.002	0.016	0.005	0.580	0.560	0.610	0.590
315	0.010	0.002	0.016	0.005	0.545	0.524	0.590	0.560
310	0.008	0.002	0.013	0.000	0.470	0.450	0.525	0.490
305	0.007	0.000	0.013	0.000	0.375	0.360	0.425	0.395
300	0.005	0.000	0.010	0.000	0.283	0.260	0.325	0.300
290	0.002	0.004	0.007	0.003	0.135	0.135	0.156	0.142
280	0.000	0.070	0.004	0.005	0.060	0.130	0.069	0.060
270	0.000	0.067	0.004	0.007	0.040	0.110	0.045	0.037
260	0.010	0.027	0.007	0.003	0.070	0.095	0.060	0.053
250	0.015	0.033	0.018	0.010	0.123	0.145	0.120	0.120
240	0.030	0.140	0.044	0.044	0.154	0.225	0.190	0.190

\* This material was an ammonium salt containing 3.5 per cent moisture. The absorption densities have been corrected to an anhydrous material and to a sodium salt to make it comparable with the other penicillins.

0.0200 at 5  $\gamma$  per ml. to 0.0227 at about 25  $\gamma$  per ml., or about 12 per cent over this range.

*Precision and Reproducibility*—An examination of the results in Tables III and IV will show how readily reproducible the results are. Occasionally a value is obtained which is out of line, for which no adequate explanation can be given. This is annoying but not a frequent occurrence.

It may be seen from the results in Tables III and IV that individual values deviate from the average rarely more than 5 per cent.

*Commercial or Crude Preparations and Interfering Substances*—Whereas the purified preparations showed practically no absorption between 290 and 400  $m\mu$  before heating, the crude or commercial preparations absorb considerably over this range (see Fig. 3). However, the increase in absorption upon heating is almost directly proportional to the penicillin concentration (see Table IV and Fig. 3). The shape and location of the curves obtained by plotting the increase in absorption against the wave-length are very close to, if not identical with, those of pure penicillin. This is

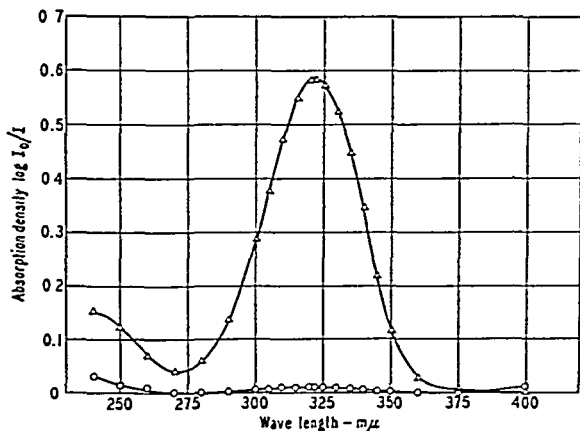


FIG. 1. Ultraviolet absorption curves of 25  $\gamma$  per ml. of crystalline sodium penicillin G, before (O) and after ( $\Delta$ ) heating 15 minutes at 100° in 0.3 M acetate buffer, pH 4.6.

strong evidence that only penicillin is responsible for the change in absorption density upon heating.

Table IV shows the results of two series of spectrophotometric analyses of five different commercial preparations carried out on different days. Bioassays<sup>3</sup> and Scudi colorimetric analyses<sup>4</sup> of the samples were included

<sup>3</sup> The bioassays were obtained by a slightly modified Oxford staphylococcus cup method (2) (an unpublished modification from The Squibb Institute for Medical Research and the Biological Laboratories of E. R. Squibb and Sons). The figures are the average of four to eight single assays with six cups per assay. They were made by Miss Helen Jones of the Penicillin Potency Laboratory of E. R. Squibb and Sons, New Brunswick, New Jersey.

<sup>4</sup> The colorimetric values (Scudi, unpublished method) represent the average of six to seven analyses of each sample. They were determined in Dr. A. P. Richardson's Pharmacological Division of The Squibb Institute for Medical Research.

for comparison. It may be seen that the spectrophotometric analyses are in substantial agreement with those obtained by the two other methods.

TABLE III

*Change in Absorption Density at 322 m $\mu$  after Heating Various Concentrations of Crystalline Sodium Penicillin G*

	Absorption densities, $\log \frac{I_0}{I}$			
	Before	After	$\Delta$	$E_{1\text{ cm.}}^{1\%}$
$\gamma$ per ml.				
2.5	0.000	0.053	0.053	0.0212
2.5	0.000	0.050	0.050	0.0200
2.5	0.004	0.054	0.050	0.0200
2.5	0.002	0.050	0.048	0.0192
5.0	0.000	0.105	0.105	0.0210
5.0	0.005	0.102	0.097	0.0198
5.0	0.005	0.098	0.103	0.0206
5.0	0.000	0.100	0.100	0.0200
7.5	0.000	0.143	0.143	0.0191
7.5	0.005	0.157	0.152	0.0203
10.0	0.000	0.210	0.210	0.0210
10.0	0.003	0.215	0.212	0.0212
12.5	0.002	0.267	0.265	0.0212
12.5	0.003	0.270	0.267	0.0213
15.0	0.003	0.342	0.339	0.0226
15.0	0.004	0.332	0.328	0.0218
20.0	0.000	0.445	0.445	0.0223
20.0	0.004	0.449	0.445	0.0223
25.0	0.008	0.572	0.564	0.0226
25.0	0.013	0.605	0.592	0.0236
30.0	0.013	0.695	0.682	0.0227
30.0	0.018	0.700	0.682	0.0227
35.0	0.015	0.807	0.792	0.0226
35.0	0.016	0.825	0.809	0.0231
40.0	0.020	0.925	0.905	0.0226
40.0	0.025	0.938	0.913	0.0228
50.0	0.030	1.13	1.10	0.0220
50.0	0.035	1.16	1.125	0.0225
75.0	0.038	1.70	1.66	0.0221
75.0	0.048	1.78	1.74	0.0232

Thus far no impurity has been found whose absorption at 322 m $\mu$  is changed on heating under the conditions of the method. Crystalline or commercial impure penicillins inactivated by 0.1 N HCl or 0.1 N NaOH do not show any increase in absorption when subsequently heated and analyzed

by the present method (see Fig. 3). Thus penicillin inactivated, but carried along during purification, will not interfere with the determination.

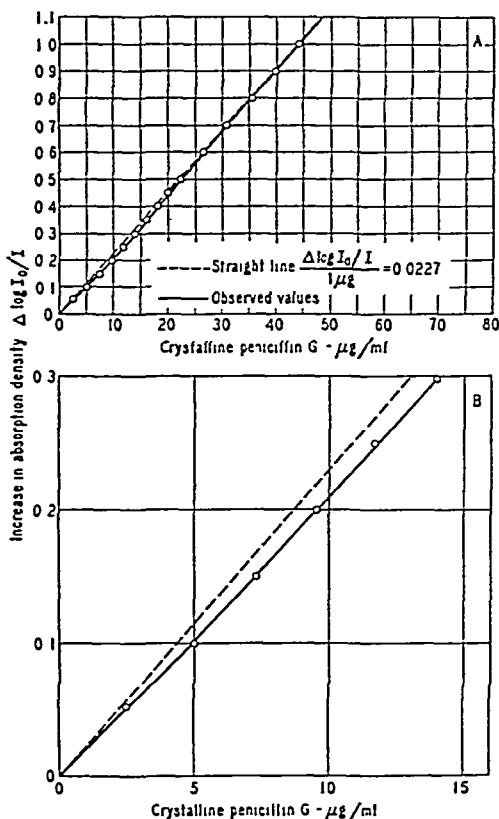


FIG. 2. Effect of increasing concentrations of crystalline sodium penicillin G on the change in absorption density at 322  $m\mu$  brought about by heating under standard conditions.

A great many substances either absorb or scatter light of 322  $m\mu$  and when present in sufficient concentration prevent a precise estimation of the penicillin. Blood, urine, and certain microbiological suspensions contain such interfering substances in high concentrations. It has not been possible as yet to separate quantitatively the penicillin from these sub-

stances and thus render the spectrophotometric method applicable to such systems.

**Extinction**—If it is assumed that the above method produces a complete conversion of penicillin into the absorbing material, then the extinction,  $E_{1\%}^{1\text{cm.}}$ , for this substance is about 227 (see Table III). The molecular extinction, assuming no change in molecular weight, would be about 8000.

Since the molecular weights (3) of the various penicillins as well as their extinctions (see Table II) do not differ by more than 10 per cent, the molecular extinctions of all these heated penicillins will be nearly the same.

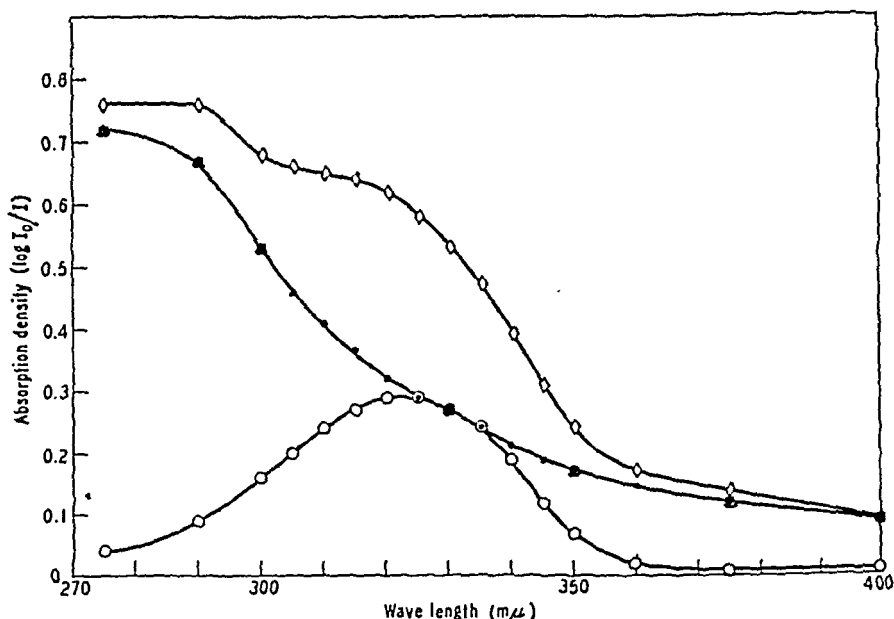


FIG. 3: The ultraviolet absorption curves of 75  $\gamma$  per ml. of a crude penicillin preparation (No. 1006, about 18 per cent penicillin).  $\bullet$  before heating;  $\diamond$  after heating;  $\square$  increase caused by heating; treated 30 minutes with 0.1 N alkali ( $\square$ ) or 0.1 N HCl ( $\triangle$ ), then neutralized, and heated in acetate, pH 4.6.

**Variables of Method and Their Effect on Absorption Density**—Figs. 4, 5, and 6 bring out the effect of most of the variables of the method on the absorption density of different penicillin preparations. Preparation 1006 was dark red and assayed only 18 per cent penicillin. Preparation 999 was light yellow and was about 60 per cent penicillin, while the crystalline sodium penicillin G was considered 100 per cent.

Other experiments indicate that unheated solutions of penicillin in 0.3 M acetate buffer, pH 4.6, change their absorption densities at the rate of 3 to 4 per cent of the heated absorption value per hour at 26°. Thus, for

TABLE IV  
Analysis of Commercial Penicillin Preparations

Preparation No.	Per cent penicillin	Dilution in water*	Absorption density, $\log \frac{I_0}{I}$			Oxford units per ml. original sample			
			Un-heated	Heated	$\Delta$	Spectrophotometric†		Bioassay, staphylococcus cup method	Spectrophotometric method
						Sample A	Sample B		
1585	44	1:1000	0.070	0.48	0.41	119,000		128,000	114,000
		1:1500	0.050	0.325	0.275		120,000		
1612	51	1:300	0.045	0.027	0.23	20,000		31,800†	15,000
		1:200	0.065	0.41	0.345		20,000		16,000
1614	78	1:600	0.050	0.44	0.39	68,000		60,300	57,000
		1:1000	0.038	0.28	0.242		70,000		
1618	43	1:600	0.060	0.355	0.295	34,000		40,000†	29,000
		1:500	0.044	0.28	0.236		34,000		31,000
1621	57	1:60	0.065	0.47	0.405	7,000		6,560	5,045
		1:100	0.040	0.29	0.25		7,250		
Crystalline sodium G		9.7 mg. in 150 ml.	0.007	0.35	0.343				
		9.7 mg. in 300 ml.	0.007	0.18	0.173				

\* This dilution does not include the 1:4 dilution in acetate buffer of Procedure A.

† The acetate buffer used in these particular analyses was pH 4.8.

‡ Since this work was completed, it has been found that these two preparations were more unstable than usual and that a number of days elapsed between performance of the bioassay and the spectrophotometric analysis.

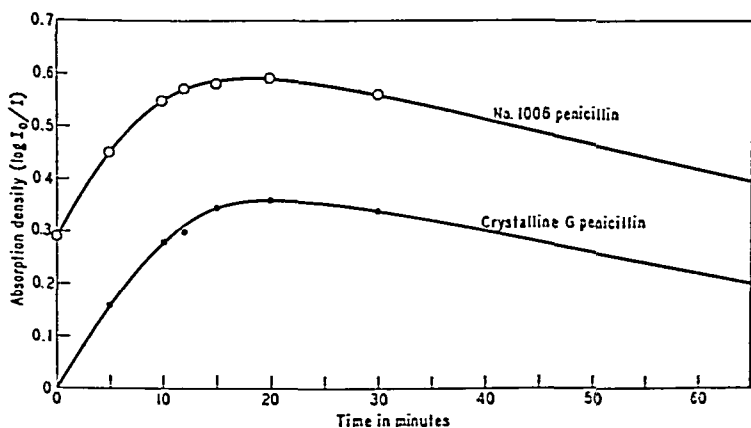


FIG. 4. Effect of time of heating at 100° in 0.3 M acetate buffer, pH 4.6, on the absorption density measured at 322 mμ.

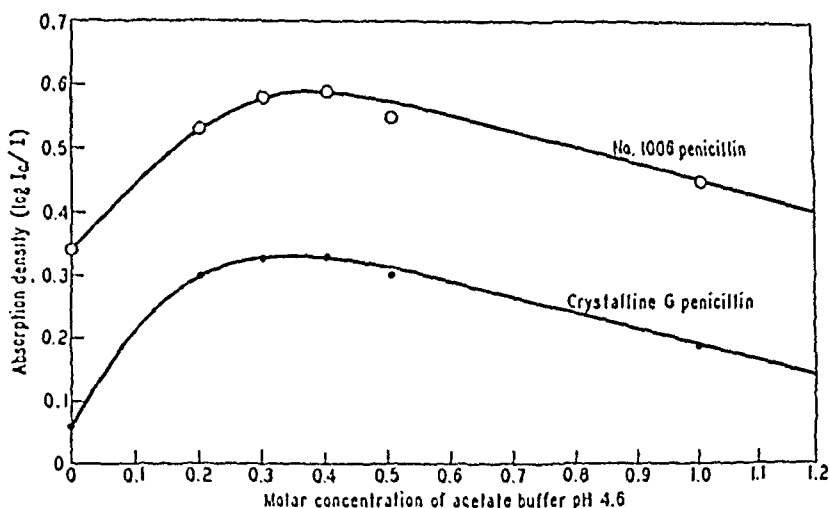


FIG. 5. Effect of concentration of acetate buffer on the absorption density of penicillin preparations at  $322 m\mu$  after heating 15 minutes at  $100^\circ$ .

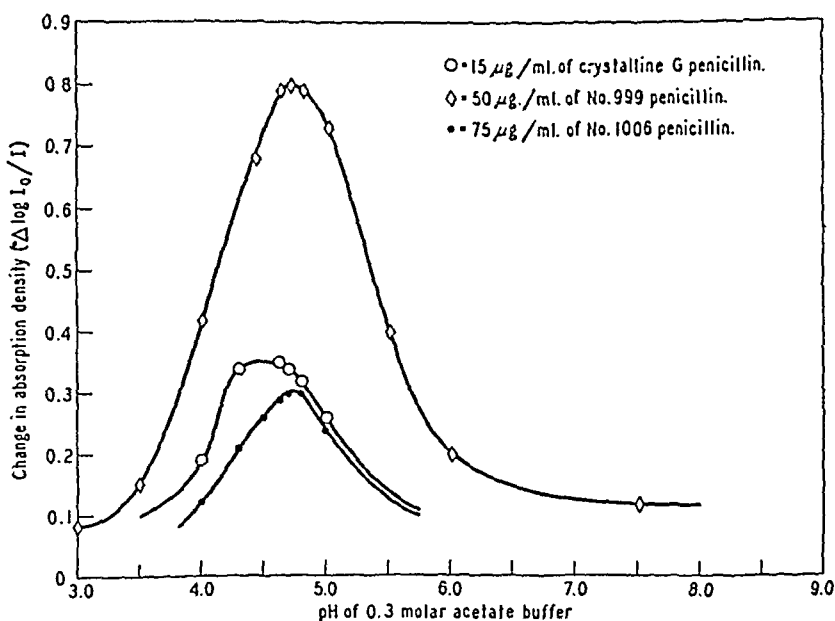


FIG. 6. Effect of pH on the change in absorption densities at  $322 m\mu$  produced by heating penicillin preparations 15 minutes at  $100^\circ$ .

at least a half hour the change is negligible. Once the samples have been heated and cooled, there is no further change for several hours.



Attempts to increase the absorption density by using solvents other than sodium acetate-acetic acid buffer of pH 4.6 were without success. The materials tested at pH 4.6 were formate, chloroacetate, glycine, propionate, lactate, citrate, butyrate, borate, phosphate, sodium octyl sulfate, as well as magnesium, barium, mercuric, and potassium acetates. Substances such as sodium chloride, magnesium sulfate, ethyl alcohol, methyl cello-solve, acetic anhydride, and others were added to 0.3 M acetate, pH 4.6, but were likewise depressing in action. Propionic acid-sodium propionate buffer at pH 4.6 was just as effective as acetate buffer but no more so.

Heating for various periods of time at 80°, or allowing the samples to stand for long periods at room temperature, failed to raise the absorption value above that obtained by heating 15 minutes at 100°.

*Apparatus*—While any sensitive ultraviolet spectrophotometer will presumably be adequate, the Beckman (1) instrument is admirably suited for this measurement. It requires only 4 ml. of solution and has proved very reliable. The instrument was standardized against the strong hydrogen line at 656  $m\mu$ , as recommended by the manufacturer, and in addition against 0.02 per cent benzene solution in isooctane. The three highest absorption maxima were obtained at 261, 245.5, and 249  $m\mu$ , which are within 1 or 2  $m\mu$  of the positions given in the International Critical Tables.

#### DISCUSSION

A discussion of the relative merits of the present method may not be out of place at this point. They are speed and simplicity, reliability and reproducibility, the wide range of concentrations included by a single test, the small volume and concentration of material required, the freedom from interference by normal impurities in commercial preparations, and independence of the nature or proportions of the various penicillins.

The biggest objection to the method is the cost of the instrument for measurement of the absorption density. A photoelectric colorimeter with quartz cells and a steady light source confined to 315 to 330  $m\mu$  could be used in place of the spectrophotometer.

The spectrophotometric method gives a precise value of the total penicillin content of any preparation. This may or may not be quantitatively related to its antibiotic activity. However, since the antibiotic activity varies with the test organism (4) and among the different penicillins (4, 5), there is no particular advantage in having the penicillin content expressed in Oxford units rather than in mg. of pure penicillin. Differential bioassays on a variety of organisms yield useful qualitative information only when the mixture of penicillins is unusual, such as when there is a high proportion of K, X, or F penicillin. Specific and quantitative tests for each different penicillin would be preferable to any test now available.

## SUMMARY

A method is described by which 5 to 75  $\gamma \approx$  about 8 to 120 Oxford units per ml., of any of the four known penicillins, pure or crude, may be determined in 20 minutes with an error not greater than  $\pm 5$  per cent.

The writer is glad to acknowledge the receipt of advice and generous quantities of crystalline, as well as crude preparations, from the individuals and organizations indicated in Table I.

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## FERRITIN

### IX. INCREASE OF THE PROTEIN APOFERRITIN IN THE GASTROINTESTINAL MUCOSA AS A DIRECT RESPONSE TO IRON FEEDING. THE FUNCTION OF FERRITIN IN THE REGULATION OF IRON ABSORPTION

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(Received for publication, May 1, 1946)

The function of ferritin in the storage of iron, especially in the organs of the liver, spleen, and bone marrow, has been previously described (1, 2). In this paper, the rôle of ferritin in the regulation of the absorption of iron by the intestinal mucosa is considered.

From recent work of McCance and Widdowson (3) and of Greenberg, Copp, and Cuthbertson (4) it has become clear that iron is handled by the body in a manner differing from that of elements like sodium or potassium. These are absorbed freely and excess is excreted. Iron, however, once absorbed or parenterally administered, is found to be very largely retained by the body. It becomes obvious that if excess iron is not to be excreted a mechanism for the control of iron absorption must be present.

That this mechanism for the regulation of iron absorption is localized in the gastrointestinal mucosa was shown by the work of Hahn, Bale, Ross, Balfour, and Whipple (5). Chronically anemic dogs were found to absorb iron at a rate 5 to 15 times the normal, indicating that, in the normal dog, some resistance or block to iron absorption was present. A sudden severe bleeding of a normal dog did not bring about an immediate response of increased iron absorption; increase began only after several days, suggesting that the resistance to iron absorption was indirectly related to the iron reserves of the body. Greater or lesser degrees of resistance to iron absorption were noted. For example, after administration of iron, the degree of resistance to further iron absorption could be shown to increase rapidly for several hours. This resistance, or "mucosal block," did not disappear immediately but remained for several days and then gradually declined. These investigators accordingly suggested that the mucosal block might be represented by a temporary storage of iron in the mucosal cells, possibly in the form of ferritin. Only when this storage iron of the mucosa had been depleted below a certain level would more iron be absorbed.

The properties of ferritin (a protein of molecular weight 465,000 to which is attached a specific type of iron hydroxide micelle) would appear to be

well suited to play such a rôle. In order to obtain evidence that ferritin is one of the important factors in the regulation of iron absorption, it is necessary to demonstrate, in the first place, that ferritin is present in the mucosa; secondly, that feeding of iron will result in a rapid increase in the ferritin content of the mucosa, parallel to the rapid development of the mucosal block; and, lastly, that the ferritin in the mucosa remains for several days and only slowly disappears in a manner parallel to the slower disappearance of the "mucosal block."

### *Methods*

Male albino guinea pigs, raised at the Institute and fed on a diet of cabbage, hay, and oats, were used. They were generally selected at a stage of rapid growth, *i.e.* at 500 to 700 gm. body weight when there appeared to be a relatively great demand for iron, so that iron absorption might be presumed to be not greatly impeded by excess storage iron. The animals were sacrificed by a sharp blow on the head. The tissues examined were the cardiac stomach, which makes up most of the stomach in the guinea pig, and the pyloric stomach, which is a small smoother area around the pyloric sphincter; in addition, the small intestine (approximately 100 cm. long) was divided arbitrarily into three equal lengths, and, in succession, the cecum, a region about 8 cm. long and 3 cm. wide, the large intestine, some 60 cm. long, consisting of a portion 1 cm. wide, and, lastly, a narrow portion about 0.5 cm. wide.

The method employed to determine ferritin in the gastrointestinal tract is only semiquantitative but has the advantage that as little as 2 to 5 cm. lengths of the tract can be used for a ferritin determination. The procedure used was to cut the tract lengthwise, wash away the intestinal contents with isotonic saline, dry the tissue superficially, and scrape off approximately 0.2 gm. portions (fresh weight) of the mucosa onto glass slides. Microscopic examination has shown that the mucosa is readily separated from the submucosa by gentle scraping. This portion was then stirred with 3 to 4 drops of 10 per cent aqueous  $\text{CdSO}_4$ , a cover-slip was placed over it, and the moisture was permitted to evaporate slowly overnight in a chamber containing some filter paper saturated with water. Complete evaporation was avoided. Crystals of ferritin appeared, the concentration being estimated by counting the crystals under a high dry or oil immersion lens. Longer periods for crystallization did not change the count significantly. When ferritin was present in high concentration, the crystals were found to be numerous and also relatively larger than in other circumstances. On the other hand, traces of ferritin were often detected only by tedious search under an oil immersion lens for tiny ferritin crystals. For example, the first evidence of the presence of ferritin in the intestinal mucosa was

obtained by finding on a slide four ferritin crystals of the size of red blood cells. The crystals are extremely insoluble when once formed. They have a tendency to develop on the under side of the cover-slip, thus greatly facilitating observation. They are readily recognized by their brownish color, high refractive index, and sharp crystalline octahedral edges. No colorless crystals were observed. To avoid giving the impression of a quantitative procedure, the content of ferritin given in Tables I and II is designated by numerals from 0 to 5. With the aid of an assistant, the dissecting, washing, and sampling of the gastrointestinal tract were accomplished in 30 to 45 minutes.

In the feeding of iron, ferrous ammonium sulfate dissolved in molasses syrup in a concentration of 10 mg. of Fe per cc. was administered by pipette. In order to determine the histological distribution of iron in the mucosa, scrapings of the mucosa were treated with a few drops of isotonic saline freshly treated with hydrogen sulfide (6), and a cover-slip was placed on the sample, which was then examined directly.

#### EXPERIMENTAL

In a normal guinea pig of around 600 gm. weight (Table I), the ferritin of the intestinal mucosa was barely detectable in a region about 5 cm. long starting just below the pyloric sphincter (*i.e.*, the duodenal region), where it was higher than anywhere else along the tract. Ferritin was only occasionally detected in other portions of the small intestine. However, in comparison with the ferritin concentration of other tissues of the guinea pig, the duodenal mucosa contained more ferritin per unit weight than did the spleen, kidney, marrow, and pancreas, but less than did the liver.

The response to the feeding of ferrous ammonium sulfate in molasses was well marked 4 to 5 hours after the feeding, the ferritin having increased in concentration especially in the mucosa of the duodenal region and also in the jejunal region. Some 7 hours after iron was fed the ferritin content had attained a maximal value and was present in the mucosa all along the small intestine. It is probable that the response of increased ferritin to iron feeding was more rapid than is indicated by these figures, since the high sugar content of the administered ferrous solution appreciably slowed down the emptying time of the stomach. At the end of 7 hours, iron was still present in the stomach, a strong positive test for ferrous iron, and a very strong positive test for ferric iron being observed with the  $\alpha, \alpha'$ -dipyridyl reagent.

The feeding of ferrous iron for several days in succession did not change the picture of ferritin distribution which was observed 7 hours after a single iron feeding. When iron was fed so as to induce maximal ferritin formation and the iron feedings were then stopped, ferritin was observed

TABLE I

*Ferritin Content of Gastrointestinal Mucosa of Guinea Pig*

The figures signify the following number of crystals per slide: 5 = 500 to 1000, 4 = 100 to 500, 3 = 50 to 100, 2 = 10 to 50, 1 = 1 to 10.

Guinea Pig No.	Treatment	Weight of guinea pig gm.	Stomach		Small intestine, approximately 100 cm. long			Cae-cum, 1 cm. long	Large intestine, 50 cm. long		Liver	Spleen
			Car-diac	Py-lo-ric	1st third	2nd third	Last third		1st half	2nd half		
1	Normal	480	0	0	1, 0, 0, 0, 0	0, 0, 0	0, 0		0		1	
2	"	790	0	0	1, 0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0	0	0	0	
3	"	650			1, 1, 0, 0, 0	0, 0, 0, 0	1, 0, 1, 0	0	0	0		
4	"	640			1, 0, 1, 0, 0	0, 1, 0, 0	0, 1, 0, 0		0	0		
5	"	400	0	0	0, 0, 0, 0	0	0		0			
Rate of appearance of ferritin after 20 mg. ferrous iron was fed												
6	1 hr. after feeding	600	0	0	1, 1, 0, 1, 1, 0	0, 0, 0, 0	0, 0, 0, 0		0	0	0	0
7	2.3 hrs. after feeding	550	0	0	1, 0, 0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0		0	0	3	0
8	4.5 " "	590	0	2	3, 1, 1, 1, 2	1, 1, 0, 0	0, 0, 0, 0		1	0	3	0
9	7 " "	480	0	2	4, 4, 2, 3	2, 2, 2, 1	2, 1, 1, 2		0	0	4	0
Effect of longer feeding of ferrous iron on ferritin content of mucosa												
10	20 hrs. after feeding 10 mg. ferrous iron	560			4, 4, 3, 2, 2, 2	1, 2, 1, 1	1, 0, 1, 0		0		5	0
11	"	560	0	1	4, 4, 2, 2, 1, 2	2, 1, 0, 0	1, 1		0	0		
12	Fed 10 mg. ferrous iron; after 17 hrs. fed another 10 mg.; examined after 23 hrs.	575	0	1	5, 5, 5, 4, 4, 3	2, 2, 1, 1	1, 2, 1	0	1	0	5	

		Rate of disappearance of ferritin from mucosa after feeding ferrous iron									
		675	1	0	4, 4, 3, 2, 2	2, 2, 2, 2	2, 0	0	0	0	0
13	Fed 10 mg. ferrous iron each day for 2 days; examined 3rd day	675	1	0	4, 4, 3, 2, 2	2, 2, 2, 2	2, 0	0	0	0	0
14	Fed 10 mg. ferrous iron each day for 3 days; examined 4th day	480	1	2	4, 3, 2, 2, 2, 1	1, 1, 1, 1	1, 1	0	1	4	1
15	Fed 10 mg. ferrous iron each day for 9 days; examined 10th day	610	2	2	3, 3, 3, 3, 2	3, 2	2, 1	1	1	1	5
16	Fed 10 mg. ferrous iron each day for 10 days; examined 11th day	610	2	2	3, 3, 3, 3, 2	3, 2	2, 1	1	1	1	5
17	Fed 20 mg. ferrous iron; examined 4th day	650	2	1	2, 2, 2, 1	1, 2, 2, 1	2, 3	0	1	4	0
18	Fed 20 mg. ferrous iron; examined 6th day	680	2	1	1, 1, 1, 1	1, 1, 1, 1	2, 1	1	1	4	0
19	Fed 20 mg. ferrous iron each day for 2 days; examined 8th day	480	0	0	2, 0, 0, 0	0, 0	0, 0	1	1	3	0
20	Fed 20 mg. ferrous iron each day for 2 days; examined 11th day	470	0	0	2, 0, 1, 0, 0, 0	1, 0, 0, 1	0, 0	0	0	3	0
21	Fed 20 mg. ferrous iron each day for 2 days; examined 13th day	670	0	0	2, 0, 0, 0, 0, 0	1, 0, 0, 0	0, 0	0	0	3	0

\* Guinea Pig 6, stomach quite distended; little iron has left the stomach. Guinea Pig 7, stomach less distended; some iron has left the stomach; the surface of duodenum staining black with  $\text{H}_2\text{S}$ . Guinea Pig 8, stomach still contains sufficient ferrous iron to give a strong dipyriddy test; strong iron reaction especially in lower portion of small intestine. The ferritin content of Guinea Pig 10 is kidney 0, pancreas 0; Guinea Pigs 12 and 16, kidney 0; Guinea Pig 13, kidney 0, marrow 0; Guinea Pig 14, kidney 1, bone marrow 0, testes 0, pancreas 0.

to have diminished appreciably by the 3rd day after the last feeding, but only by about the 6th day after the last feeding did the ferritin content of the mucosa diminish to approximately the level of the normal control animals.

With successive iron feedings (Table I, Guinea Pigs 19, 20), an accumulation of iron became evident histologically when the mucosal scrapings were treated with a saline solution containing hydrogen sulfide. The typical picture of iron staining observed was similar to that early described by Macallum (7).

In the normal unstained lamina propria of the duodenum, some large fixed tissue histiocytes were observed in the tip of the core of the villus. These cells often contained yellow or greenish yellow granules and vacuoles. When stained with hydrogen sulfide, these cells of the normal mucosa occasionally appeared somewhat darkened but only in the duodenal region, not below. On feeding iron for 5 to 7 days, the granules and vacuoles of these histiocytes were stained a dark gray to black with hydrogen sulfide, and tiny black granules were sometimes observed within the columnar epithelial cells. Not infrequently, some of these histiocytes were seen lying between the columnar cells as if preparatory to being extruded into the gut. Below the duodenal region, the reaction for iron became less pronounced and was rarely seen in the lower portion of the small intestine or in the large intestine. No correlation was observed between the feeding of iron and the histologically stainable iron of the liver and spleen.

In considering the place of origin of mucosal ferritin, two types of cells appear as possibilities; *i.e.*, the fixed tissue histiocytes and the columnar epithelial cells. Since large variations in the stainable iron of the histiocytes have been observed without corresponding changes in the ferritin content of the mucosa, it is suggested that it is the columnar epithelial cells of the mucosa which may be the site of formation of the ferritin. The fixed tissue histiocytes, on the other hand, would seem to function primarily as a protective mechanism by taking up excessive amounts of iron into their vacuoles. Continued high iron feedings appear to lead to the extrusion of these heavily iron-laden cells into the intestinal cavity.

To explain the increase of ferritin in the mucosa on feeding iron, it is necessary to consider what mechanism could be supposed to bring about an increase in the protein apoferritin. One suggestion that seems reasonable is that apoferritin (like some adaptive enzymes) is constantly being produced and broken down in the mucosal cells so that, at any one time, very little apoferritin is present. If apoferritin, when formed, was protected by the adhering iron hydroxide micelles (in a manner analogous to the protection afforded an enzyme in the presence of its substrate), it might tend to accumulate as ferritin. In this manner, ferritin might appear to increase in response to iron feeding.



In order to determine whether the increase in ferritin in the mucosa, on feeding of iron, could be related to differences in the rate of autolytic digestion of apoferritin and ferritin, the following experiments were carried out. Crude beef liver homogenate was selected as a source of proteolytic enzymes, since no ferritin can be isolated from this tissue with the customary procedure and no correction blank need therefore be made. The liver was homogenized with an equal weight of isotonic saline. To 150 cc. of this homogenate were added, with stirring, 4 cc. of horse ferritin (or

TABLE II  
*Incubation of Horse Apoferritin and Ferritin with Beef Liver Homogenate*

Experiment		Time of incubation at 40°	Packed, twice crystallized ferritin or apoferritin
		hrs.	cc.
A	4 cc. ferritin		0.13
	4 " apoferritin		0.13
	Liver brei control	0	0.00
	" " + 4 cc. ferritin	0	0.100
B	" " + 4 " apoferritin	0	0.110
	" " control	4	0.00
	" " " incubated 4 hrs.; 4 cc. ferritin added prior to isolation	0	0.050
C	Liver brei + 4 cc. ferritin	4	0.100
	" " + 4 " apoferritin	4	0.050
	" " control	8	0.00
	" " " incubated 8 hrs.; 4 cc. ferritin added prior to isolation	0	0.013
D	Liver brei + 4 cc. apoferritin	8	0.015
	" " + 4 " ferritin	8	0.020
	" " buffered with $\text{PO}_4$		
	Liver brei control incubated 20 hrs.; 4 cc. ferritin added prior to isolation	0	0.055
	Liver brei control incubated 20 hrs.; 4 cc. apoferritin added prior to isolation	0	0.061
	Liver brei + 4 cc. apoferritin	20	0.055
	" " + 4 " "	20	0.070

apoferritin) solution and 2 cc. of toluene, and the mixture was incubated for various lengths of time at 40°. A control vessel was also run in which homogenate was incubated for a given interval and then ferritin or apoferritin added just prior to isolation. For the isolation, 100 cc. of water were added to the vessel, which was heated rapidly to 80°, and the solution was filtered, treated with 35 gm. of ammonium sulfate for each 100 cc. of filtrate, and left in the ice box overnight. The precipitate was then dialyzed against water for a day and crystallized in 10 per cent  $\text{CdSO}_4$ .

The ferritin (or apoferritin) was recrystallized as previously described (8) and, finally, the crystals were centrifuged down in graduated tubes under standardized conditions. The recoveries are expressed in terms of the volume of the crystals obtained after two crystallizations. In Experiments A, B, and C, no attempt was made to control the pH of the autolysate, which decreased with time. In Experiment D, 20 cc. of  $\text{M Na}_2\text{HPO}_4$  were added at the start to the homogenate, thus bringing the pH from 6.3 up to 7.3. At the end of 20 hours incubation, the pH was 5.1.

The recovery of about 75 to 80 per cent of the ferritin, as twice crystallized material, from a freshly prepared homogenate containing 40 mg. of ferritin and 75 gm. wet weight of liver (Table II, Experiment A) illustrates the adequacy of the isolation procedure and the insolubility of the crystals. Incubation at 40° with liver homogenate for a period of more than 4 hours led to somewhat lower recoveries. These low recoveries were due primarily to difficulties arising from the separation of ferritin (or apoferritin) from the products of the liver autolysate, as shown by corresponding control experiments.

Under the conditions of these experiments, there arose an anaerobic, actively reducing medium, and a pH tending toward 5, which greatly favored catheptic activity and the breakdown of proteins. However, there was no difference in the rate of disappearance of the protein apoferritin as compared to ferritin, and no appreciable autolysis of either of these substances can be considered to have occurred.

In another experiment, in which an active trypsin preparation was incubated for 2 days at 38° in phosphate buffer at pH 7.5, little or no digestion of native apoferritin was observed. Heat-denatured samples of ferritin and apoferritin materials, however, were found to be digested to the extent of over 90 per cent (non-precipitable by trichloroacetic acid).

From these experiments it is suggested that there may be other mechanisms than the ordinarily considered proteolytic enzymes which may be responsible for the digestion of apoferritin in the living mucosal cells.

#### DISCUSSION

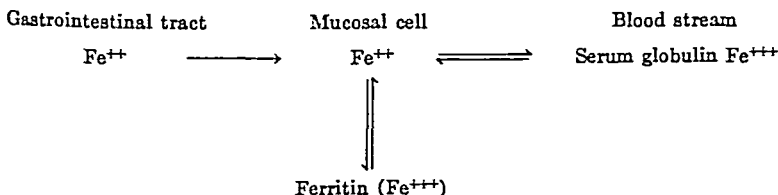
The experimental data presented here, when considered together with the data of Hahn *et al.* (5), suggest that the regulatory mechanism controlling iron absorption is a function of the ferritin content of the mucosa. There is good evidence that iron is absorbed in the ferrous form. When iron is fed, the excess iron absorbed is converted to the ferric state and is temporarily stored in the form of ferritin in the mucosa.

In order to explain the regulatory function of ferritin, the following hypothesis, modified in part from that of Hahn *et al.* (5), is suggested (see the accompanying diagram). When the cell is "physiologically saturated" with respect to ferrous iron, no further iron is assumed

to be absorbed from the tract. An equilibrium relationship is postulated between the ferric iron of ferritin and the ferrous iron in the mucosal cells. After an iron feeding, ferrous iron enters the mucosal cells, much of it being temporarily stored in the form of ferritin. A point is reached eventually at which the cells would be "physiologically saturated" with respect to ferrous iron and then no further iron would be absorbed. Experiments indicate that it requires several hours to attain this saturation state, and, during this time, a resistance or mucosal block to further iron absorption develops. Parallel to this increase in resistance is the rapid increase in the content of ferritin. Then the iron, perhaps in the ferrous form, diffuses out into the blood stream, where it is immediately autoxidized to the ferric state and transported away, attached to some specific component of the ( $\beta + \gamma$ ) globulin fraction (9). The diffusion of iron out of the cell would involve the reduction of some ferric iron of ferritin whereby the saturation state of ferrous iron would be maintained. Only when the ferritin iron had decreased to a point where the mucosal cells were no longer physiologically saturated with ferrous iron would more iron be absorbed from the gut. Experiment shows that only about 3 to 6 days after an iron feeding does the ferritin in the mucosa drop back almost to normal. This slower rate of disappearance of ferritin also parallels the slower rate of disappearance of the mucosal block.

What the "physiological saturation" level with respect to ferrous iron is, how the storage iron of the body or the serum iron level influences the rate of absorption, how the iron moves only in one direction through the epithelial wall are problems that await further study.

The processes outlined above are represented in the accompanying diagram.



As has been previously remarked, the absorption of iron leads to a marked increase in the ferritin of the mucosa. For example, the normal duodenal mucosa of the guinea pig may show from one to ten tiny crystals per slide, the crystals being visible only with the aid of an oil immersion objective. Some 7 hours after feeding iron, the duodenal mucosa will show 200 to 500 crystals per slide, readily counted under a high, dry objective. Since ferritin consists of the protein apoferritin to which are attached iron hydroxide micelles, it might be considered that the feeding of iron merely resulted in the attachment of the iron micelles to the apoferritin already

present within the mucosal cells. This, however, is not the case. Apoferritin has been shown to crystallize as readily as ferritin (10), the crystallization with  $\text{CdSO}_4$  being a function of the protein itself. In all of the present experiments, when ferritin could not be detected, apoferritin could not be observed either. One is thus forced to the conclusion that the feeding of iron leads to an increase in the concentration of the specific protein apoferritin within the mucosal cells of the gastrointestinal tract. The apoferritin then serves as a repository for the iron hydroxide micelles.

#### SUMMARY

A semiquantitative method has been developed which permits a relative estimation to be made of the ferritin content of 2 to 5 cm. segments along the gastrointestinal tract of the guinea pig.

Ferritin is just detectable in the duodenal mucosa but rarely elsewhere along the tract. When ferrous iron is fed, the ferritin increases markedly, especially in the duodenal and jejunal regions, an appreciable increase being noted within 4 to 5 hours, and a maximal response within 7 hours after feeding. After a period of some 3 to 6 days, the ferritin has returned to the level of the controls. These data, when considered together with radioactive iron absorption studies, suggest that ferritin in the mucosa is concerned with the regulation of iron absorption and represents the primary factor for the mucosal block. The feeding of iron leads to the increase in the concentration of the specific protein apoferritin, which appears in the form of ferritin.

Incubation with beef liver macerate did not appear to cause the autolysis of either ferritin or apoferritin, suggesting that catheptic enzymes may not be the agents which function in the disappearance of these proteins.

We wish to acknowledge our gratitude to Dr. L. Michaelis for his advice and interest in these studies.

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# PHOSPHORUS COMPOUNDS IN ANIMAL TISSUES

## III. A COMPARISON OF METHODS FOR THE ESTIMATION OF NUCLEIC ACIDS

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Recently two methods for the estimation of nucleic acids in animal tissues appeared almost simultaneously (3, 4). Although the techniques employed in these independently conducted experiments differed considerably, the results agreed remarkably well. Some discrepancies in the results obtained by the two methods were, however, evident. For this reason and because of the increasing interest in the rôle of nucleic acids in cellular physiology, it seemed of interest to compare the results obtained by the two methods on the same sample of tissue.

The present report describes the results of the analysis of six rat tissues for nucleic acids by the two methods and also describes an improved method for nucleic acid analysis which incorporates the principles of both methods.

### *Methods and Materials*

*Preparation of Tissues*—Adult rats of the Sprague-Dawley strain were used for analysis. The animals were killed by decapitation and the tissues were removed immediately and chilled on cracked ice. Portions of the tissue were weighed and homogenized in distilled water at 0° in the apparatus of Potter and Elvehjem (2).<sup>1</sup>

*Removal of Acid-Soluble Phosphorus Compounds and Phospholipides*—Equal aliquots of the tissue homogenates were pipetted into each of two 15 ml. centrifuge tubes and acid-soluble compounds were removed as described previously (4). Phospholipides were then removed from the tissue residues by the method reported previously (4) with the exception that the extractions with boiling alcohol-ether were omitted, since it was found that these extractions did not appreciably increase the yield of phosphorus extracted.

*Nucleic Acid Removal, Method of Schneider (4)*—The residue contained in one of the tubes after removal of acid-soluble and fat-soluble compounds

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<sup>1</sup> Now obtainable from the Central Scientific Company.

was heated with 5 per cent trichloroacetic acid to extract the nucleic acids (see (4)). This extract will be referred to as Fraction I. The protein residue which remained after heating with trichloroacetic acid was dissolved in 2 per cent NaOH to form Fraction II (see (4)).

*Nucleic Acid Removal, Modification of Method of Schmidt and Thannhauser (8)*—The method of Schmidt and Thannhauser was modified slightly to include heating with trichloroacetic acid to obtain the desoxyribose nucleic acid (DNA) in solution so that it might be determined colorimetrically. The procedure was as follows: The tissue residue which remained in the second centrifuge tube, after removal of acid- and alcohol-soluble materials, was treated for 20 hours at 37° with 1 N KOH (see (3)). This treatment resulted in solution of the tissue (Fraction III). DNA and protein were precipitated with acid as described (3) and the filtrate containing the pentose nucleic acid (PNA) and protein phosphorus was removed and termed Fraction IV. The precipitate containing the DNA was heated with trichloroacetic acid (see (4)) to dissolve the DNA. The trichloroacetic acid extract was designated Fraction V.

*Analyses*—Total phosphorus was determined by the method of LePage and Umbreit (1). DNA was measured by the reaction with diphenylamine (4) in Fractions I and V. PNA was measured in Fractions I and IV by the orcinol reaction (4). Nucleic acids served as standards for both reactions (see (4)). Inorganic phosphorus was precipitated from Fraction IV as the calcium salt and determined colorimetrically (1).

### Results

The analytical results are reported in Table I. In confirmation and extension of the previous results (4, 5), it was found that all of the phosphorus present in the extracts of tissues heated with trichloroacetic acid could be accounted for by nucleic acids as measured by pentose determinations (see Table I, compare Fraction I-A plus I-B with I-C).

The DNA values found by the method of Schmidt and Thannhauser (see Fraction IV-D, Table I) were higher than the results reported earlier by these authors (3). The most striking case was that of spleen in which the DNA content was found to be almost 3 times as high as they had reported. A possible explanation of the higher results we obtained may lie in the fact that our analyses include four animals, while the results of Schmidt and Thannhauser apparently were based on single analyses. With the exception of liver and brain, the DNA found by the method of Schmidt and Thannhauser (phosphorus analysis) agreed remarkably well with the DNA found by desoxyribose analyses (compare Fractions IV-D and V-A). The DNA found by desoxyribose determinations in the modified method is in excellent agreement with the DNA found by the method of Schneider



(compare Fractions V-A and I-A), indicating that the DNA has been effectively separated in the modified method.

The PNA values found by the method of Schmidt and Thannhauser were somewhat lower than the results reported by these authors (see Fraction IV-B, Table I, and (3)). The PNA found by pentose determinations was in fair agreement with the PNA calculated as described by Schmidt and Thannhauser (compare Fractions IV-C and IV-B) and in good agreement with the PNA found in the trichloroacetic acid extraction method (compare Fractions IV-C and I-B).

#### DISCUSSION

The observation that the amounts of nucleic acids found by pentose determinations in the modified method of Schmidt and Thannhauser and in the method of Schneider are the same indicates that DNA and PNA have been effectively separated in the former method. This finding indicates that the choice of the method to be used in the measurement of nucleic acids will depend upon whether a separation of the nucleic acids is necessary. If a separation of DNA and PNA is required, as would probably be the case in work with isotopes, the method of Schmidt and Thannhauser as modified in this paper is the method of choice. On the other hand, if such a separation is unnecessary, the trichloroacetic acid method can be expected to afford a simple, rapid, and accurate means of extraction and estimation of nucleic acids. In connection with the choice of methods, it should be recalled that in the trichloroacetic acid method of Schneider (4) a correction must be applied for the reaction of DNA in the orcinol reaction. Such a correction is unnecessary in the modified method, since the nucleic acids have been effectively separated.

The use of pentose determinations for the measurement of nucleic acids has been criticized on the ground that not all of the pentose in the nucleic acids reacts in the colorimetric methods (3). Our own results (Table I, and (4, 5)), in which nucleic acids were used as standards in the colorimetric reactions, show that the use of pentose is a valid procedure for the determination of nucleic acids, since in all of the tissues studied the nucleic acid found by pentose determinations agreed well with the nucleic acids as measured by phosphorus determinations. Obviously, it is a sound practice not to rely upon either pentose or phosphorus analyses alone but to employ both. A still better procedure would be to determine the phosphorus, the pentose, and the purine and pyrimidine bases simultaneously in order to arrive at a correct estimate of the nucleic acid content of the tissue. The nitrogenous bases could probably be measured by the use of ultraviolet spectrophotometry.



## SUMMARY

1. The method of Schmidt and Thannhauser for the determination of nucleic acids was modified by extracting the DNA with trichloroacetic acid so that the DNA could be determined colorimetrically.

2. The following rat tissues were analyzed by the modified method and by the method of Schneider: pancreas, thymus, liver, kidney, spleen, and brain.

3. Both methods yielded essentially the same results when the nucleic acid estimations were based on pentose determinations. Results based upon phosphorus determinations were less consistent and reliable. The need for making both types of measurements was emphasized.

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# THE SPECIFICITY OF CARBOXYPEPTIDASE

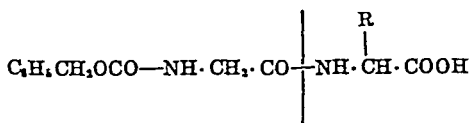
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Previous communications from this laboratory have called attention to the fact that the action of the proteolytic enzymes is characterized by a high degree of specificity (1, 2). Each enzyme hydrolyzes only such peptide bonds as are present in the substrate in a certain structural setting and, consequently, the nature of the requisite structural attributes of the substrate may be used to characterize the specificity of the enzyme. It has been found that, in addition to the indispensable groups within the peptide chain, each proteolytic enzyme requires the presence, in the substrate, of a certain type of side chain (designated R in the formula below) in a precisely defined location.

*Side Chain Specificity of Carboxypeptidase*—In the present communication, studies are presented on the side chain specificity of crystalline pancreatic carboxypeptidase. The substrates which were employed were, in general, of the following type.



Carbobenzoxylglycylamino acid

The dotted line denotes the point of enzymatic cleavage. A quantitative study was made of the kinetics of the action of carboxypeptidase on a series of substrates in which the group R was varied. In these substrates, the terminal amino acid was *L*-phenylalanine, *L*-tyrosine, *L*-leucine, *L*-isoleucine, *L*-alanine, aminoisobutyric acid, or glycine. In the case of all of these substrates, with the exception of carbobenzoxylglycylglycine, it was found that the rates of hydrolysis follow the kinetics of a first order reaction under the conditions employed in these experiments. The rate of hydrolysis per unit of enzyme concentration is given by the proteolytic coefficient *C* which has been defined as the rate constant when the enzyme concentration is 1 mg. of protein nitrogen per ml. of test solution (3, 4).

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The hydrolysis of carbobenzoxyglycylglycine was so slow that a satisfactory rate constant could not be obtained.

The data presented in Table I show that, while all of the above substrates were hydrolyzed by the carboxypeptidase preparation, they differ greatly in their sensitivity to enzymatic attack. Thus, to achieve comparable rates of hydrolysis of carbobenzoxyglycyl-L-alanine and carbobenzoxyglycyl-L-phenylalanine, 340 times as much enzyme were required for the alanine-containing substrate as was necessary for the phenylalanine-containing substrate. It may be estimated from the extent of the hydroly-

TABLE I  
*Action of Crystalline Carboxypeptidase on Synthetic Substrates*

Substrate*	Enzyme concentration, N per ml. test solution	pH	K†	C‡
	mg.			
Carbobenzoxyglycyl-L-phenylalanine (5)....	0.0002	7.3	0.0026	13
Carbobenzoxyglycyl-L-tyrosine (6).....	0.0004	7.4	0.0025	6.2
Carbobenzoxyglycyl-L-leucine.....	0.0006	7.3	0.0016	2.6
Carbobenzoxyglycyl-L-isoleucine.....	0.0025	7.3	0.00135	0.54
Carbobenzoxyglycyl-L-alanine (7).....	0.040	7.3	0.0015	0.038
Carbobenzoxyglycylaminoisobutyric acid (8).....	0.160	7.3	0.0021	0.013
Carbobenzoxyglycylglycine (9).....	0.320	7.2	0.00078§	0.0024§
Acetyldehydrophenylalanyl-L-phenylalanine (10).....	0.080	7.2	0.0025	0.032
Acetyldehydrophenylalanyl-L-leucine (11)...	0.320	7.2	0.0022	0.0069

\* The figures in parentheses represent the bibliographic references.

$$\dagger K = \frac{1}{\text{min.}} \log \frac{100}{100 - \% \text{ hydrolysis}}$$

$$\dagger C = \frac{K}{\text{mg. protein N per ml. test solution}}$$

§ Calculated on the basis of the extent of hydrolysis within 5 hours.

sis of carbobenzoxyglycylglycine within 5 hours that about 5000 times as much enzyme would be required for this substrate as for carbobenzoxyglycyl-L-phenylalanine to give comparable rates of hydrolysis.

Neither carbobenzoxyglycylsarcosine (12) nor carbobenzoxyglycyl-L-proline (12) was hydrolyzed by carboxypeptidase, even at very high enzyme concentrations (0.3 to 0.5 mg. of protein nitrogen per ml. of test solution). This indicates that the presence of the "peptide hydrogen" in the substrate is essential for the action of carboxypeptidase (*cf.* also (13)).

It was of interest to find that the unsaturated compound carbobenzoxyglycyldehydrophenylalanine (10) was resistant to the action of carboxypeptidase at an enzyme concentration of 0.16 mg. of protein nitrogen per ml. of test solution. This is in distinct contrast to the behavior of the *l* form of the saturated peptide which, as will be noted in Table I, is the most sensitive of the substrates studied.

Both acetyldehydrophenylalanyl-*l*-phenylalanine and acetyldehydrophenylalanyl-*l*-leucine were hydrolyzed by carboxypeptidase. It should be noted that each of the two acetyldehydrophenylalanyl peptides requires approximately 400 times as much enzyme for the same rate of hydrolysis as the corresponding carbobenzoxyglycyl peptides, carbobenzoxyglycyl-*l*-phenylalanine and carbobenzoxyglycyl-*l*-leucine. Thus, the exchange of the acetyldehydrophenylalanyl portion of the peptide for the carbobenzoxyglycyl portion decreased the rate of enzymatic hydrolysis to the same degree for the phenylalanine- and leucine-containing substrates. This result is similar to that noted previously for other substrates of carboxypeptidase (4).

*Antipodal Specificity of Carboxypeptidase*—It was found that, in order for carboxypeptidase to act on a carbobenzoxyglycylamino acid, the terminal amino acid must be of the *l* configuration. Thus, carbobenzoxyglycyl-*d*-phenylalanine<sup>1</sup> and carbobenzoxyglycyl-*d*-leucine (14), whose *l* antipodes are extremely sensitive to carboxypeptidase action, are resistant to the action of the enzyme. This finding is in agreement with the "poly-affinity theory" (7) of the action of proteolytic enzymes. In this theory it has been assumed that an enzyme exhibiting antipodal specificity must contain several active groups in a fixed asymmetric position. For hydrolysis to occur, these active groups must approach the essential groups in the peptide chain of the substrate. In the case of the *l* antipodes, the location of the side chain is such that the formation of the enzyme-substrate complex is unhindered. It is assumed that in the case of the *d* antipodes, however, the side chain blocks the approach of the enzyme.

*Enzymatic Homogeneity of Carboxypeptidase*—In view of the wide differences in sensitivity exhibited by the substrates listed in Table I, it seemed desirable to ascertain whether the hydrolysis of several of these substrates is effected by a single enzyme or by a number of closely related enzymes present in our sample of crystalline carboxypeptidase. To this end, four substrates of varying sensitivity were selected for study. The substrates employed were carbobenzoxyglycyl-*l*-phenylalanine, carbobenzoxyglycyl-*l*-tyrosine, carbobenzoxyglycyl-*l*-leucine, and carbobenzoxyglycyl-*l*-alanine.

<sup>1</sup> Stein, W. H., unpublished experiments.

Experiments were performed in which a solution of the enzyme was inactivated partially by heat (52°) or by alkali (pH 10). The denatured protein was removed by filtration and the filtrates were allowed to act on each of the four substrates mentioned above. If crystalline carboxypeptidase contained several enzymes, it might have been expected that the

TABLE II

*Partial Inactivation of Crystalline Carboxypeptidase by Heat and by Alkali*

An aliquot of a solution (A) of four times crystallized carboxypeptidase containing 1.077 mg. of protein N per ml. in 18 per cent NaCl was heated at 52° for 6 minutes, cooled, and filtered. The filtrate (B) contained 0.618 mg. of protein N per ml. Another aliquot of Solution A was heated for 10 minutes, cooled, and filtered. The filtrate (C) contained 0.316 mg. of protein N per ml. A third aliquot of Solution A was adjusted to pH 10.0 with 0.1 N NaOH and allowed to stand at 25° for 45 minutes. The NaOH was then neutralized with an equivalent quantity of 0.1 N HCl and the mixture was filtered. The filtrate (D) contained 0.666 mg. of protein N per ml. after correction for the slight change in volume. These solutions were then diluted and tested for activity toward the four substrates listed below.

Substrate	Enzyme solution	Enzyme concentration, protein N per ml. test solution	pH	$\kappa$	C
		mg.			
Carbobenzoxymethyl-L-phenylalanine	A	0.0002	7.3	0.0026	13
	B	0.0002	7.3	0.0025	12
	C	0.0002	7.3	0.0026	13
	D	0.0002	7.3	0.0027	13
Carbobenzoxymethyl-L-tyrosine	A	0.0004	7.4	0.0025	6.2
	B	0.0004	7.4	0.0021	5.3
	C	0.0004	7.4	0.0021	5.3
	D	0.0004	7.4	0.0024	6.0
Carbobenzoxymethyl-L-leucine	A	0.0006	7.3	0.0016	2.6
	B	0.0006	7.3	0.0014	2.3
	C	0.0006	7.3	0.0015	2.5
	D	0.0006	7.3	0.0016	2.6
Carbobenzoxymethyl-L-alanine	A	0.04	7.3	0.0015	0.038
	B	0.04	7.3	0.0015	0.038
	C	0.04	7.3	0.0015	0.038
	D	0.04	7.3	0.0016	0.040

relative rates of hydrolysis of these four substrates would have been different for the untreated and the partially inactivated enzyme solutions. As will be noted from the data in Table II, no such differences were observed, and consequently, there is no reason to assume that the hydrolysis of the four substrates is performed by different enzymes.

*Inhibition of Carboxypeptidase Action*—The apparent enzymatic homo-

geneity of crystalline carboxypeptidase noted above raises the question whether the great differences found for the sensitivity of the various carbobenzoxyglycylamino acids toward this enzyme are due to differences in the affinity of these compounds for the enzyme. For this reason,

TABLE III

*Inhibition of Crystalline Carboxypeptidase*

The rate of hydrolysis of carbobenzoxyglycyl-L-phenylalanine (0.05 M) was followed in the presence of an equivalent concentration of added substance. The enzyme concentration was 0.0002 mg. of protein nitrogen per ml. in all cases.

Added substance	pH	Time		K	Inhibition
		hrs.	per cent		per cent
None	7.3	1.0	30	0.0026	
		2.0	49	0.0025	
		3.0	66	0.0026	
		4.0	77	0.0026	
Carbobenzoxyglycyl-L-alanine	7.3	1.0	15	0.0012	
		2.0	30	0.0013	
		3.0	44	0.0014	
		4.0	53	0.0014	50
Carbobenzoxyglycylglycine	7.2	1.5	19	0.0010	
		2.0	26	0.0011	
		3.0	37	0.0011	
		4.0	49	0.0012	53
Carbobenzoxyglycylsarcosine	7.2	1.5	33	0.0019	
		2.5	46	0.0018	
		3.0	53	0.0018	
		3.5	60	0.0019	23
Carbobenzoxyglycyldehydro-phenylalanine	7.3	1.0	20	0.0016	
		2.0	35	0.0016	
		3.0	47	0.0015	
		4.0	58	0.0016	40
Carbobenzoxyglycyl-D-phenylalanine	7.3	1.0	14	0.0011	
		2.0	23	0.0010	
		3.0	34	0.0010	
		4.0	45	0.0011	60
Carbobenzoxyglycyl-D-leucine	7.4	1.5	25	0.0014	
		2.0	31	0.0014	
		3.0	40	0.0012	
		3.5	45	0.0012	50

experiments were performed in which there was studied the inhibitory effect of relatively resistant peptides on the enzymatic cleavage of a substrate which is hydrolyzed rapidly. Substrate mixtures containing equivalent concentrations of carbobenzoxyglycyl-L-phenylalanine (which is hydrolyzed rapidly) and a related resistant peptide were subjected to

the action of crystalline carboxypeptidase. The resultant hydrolysis rate was compared with that found in the absence of the resistant peptide. The data are presented in Table III.

In the absence of added resistant peptide, carbobenzoxyglycyl-*L*-phenylalanine is split rapidly to give a first order velocity constant of 0.0026 under the experimental conditions employed. In the presence of carbobenzoxyglycyl-*L*-alanine, however, the rate of hydrolysis is reduced to a constant of 0.0013, or 50 per cent of the original value. (At the enzyme concentration used in these experiments, there is no significant hydrolysis of carbobenzoxyglycyl-*L*-alanine in 4 hours.) Similarly, the addition of carbobenzoxyglycylglycine resulted in a rate which was 42 per cent of the original value. The addition of carbobenzoxyglycylsarcosine or carbobenzoxyglycyldehydrophenylalanine, neither of which is hydrolyzed by carboxypeptidase, resulted in 28 and 40 per cent inhibition respectively. Thus, the presence of certain peptides which are either completely resistant to the action of the enzyme or are split but slowly by carboxypeptidase may markedly reduce the rate of hydrolysis of carbobenzoxyglycyl-*L*-phenylalanine by this enzyme.

It was of interest to find that the addition of carbobenzoxyglycyl-*D*-phenylalanine or carbobenzoxyglycyl-*D*-leucine also resulted in inhibitions of a similar magnitude. Analogous inhibition of the hydrolysis of the *L* antipode by the *D* form has been noted for chymotrypsin (15), and for a peptidase from swine intestinal mucosa (16). These results emphasize the dangers inherent in kinetic studies in which *DL* mixtures are used as substrates.

The data presented in Table III, while suggestive, require cautious interpretation. According to the polyaffinity theory, the combination of a substrate of carboxypeptidase with the active center of the enzyme involves at least two structural elements of the substrate, the sensitive peptide linkage and the free carboxyl group. It may well be that some of the inhibitory peptides (such as carbobenzoxyglycyl-*L*-alanine) compete with the sensitive peptides for the enzyme by combining with the active catalytic center in the manner postulated in the polyaffinity theory. If this is the case, then the similarity in the observed extent of inhibition would suggest that the peptides listed in Table III have similar affinities for the active center of carboxypeptidase. The difference in the rates of hydrolysis of peptides which are split by carboxypeptidase would then be due to the difference in the rates at which the respective enzyme-substrate complexes decompose to form the hydrolytic products. This interpretation cannot, however, apply to the inhibitions by the *D* peptides or carbobenzoxyglycylsarcosine, if the polyaffinity theory is to be maintained. In these latter cases, it would be necessary to assume that the inhibitor



combines with the active center of the enzyme at only one point, possibly through the carboxyl group of the inhibitor. It is also possible that the inhibition by *d* peptides may also involve association with the corresponding sensitive *l* antipode, thus reducing the effective concentration of the latter. Studies on the quantitative relationships between substrate concentration and the rate of carboxypeptidase action, which are in progress, should throw more light on this question.

#### EXPERIMENTAL

*Carbobenzoxylglycyl-l-leucine*—13.1 gm. of carbobenzoxylglycyl chloride dissolved in 100 ml. of dry ether were added to an ice-cold ether solution of *l*-leucine methyl ester (prepared from 9.8 gm. of the hydrochloride). The acid chloride was added in two portions, the second being followed by 75 ml. of saturated aqueous potassium bicarbonate. The cold reaction mixture was shaken for 30 minutes, then allowed to stand at room temperature for 1 hour. 2 ml. of pyridine were added and the mixture shaken for 5 minutes. The ether layer was removed and washed with dilute hydrochloric acid, bicarbonate, and water and then dried. The ether was removed and the oily ester was saponified by dissolving in 200 ml. of ethanol and adding 54 ml. of 1.0 *N* sodium hydroxide. After 30 minutes the reaction mixture was acidified to Congo red with *N* hydrochloric acid and concentrated under reduced pressure until an oil began to separate. The oil crystallized on standing at 4° overnight. The crystals were collected and the mother liquors concentrated to obtain a second crop. The combined yield was 11.3 gm. The crude product was recrystallized from a mixture of ethyl acetate and petroleum ether; m.p. 141–142°.

$C_{18}H_{22}O_5N_2$ .	Calculated.	C 59.6, H 6.9, N 8.7
322.4	Found.	" 59.8, " 6.9, " 8.7
$[\alpha]_D^{25} = -10.3^\circ$ (5% in ethyl alcohol)		

*Carbobenzoxylglycyl-l-isoleucine*—This compound was prepared from *l*-isoleucine methyl ester by a procedure analogous to that used to prepare carbobenzoxylglycyl-*l*-leucine. The *l*-isoleucine methyl ester was prepared by esterifying *l*-isoleucine with methanol and HCl in the usual way and then liberating the free ester from the oily methyl ester hydrochloride with excess sodium hydroxide. From 10 gm. of *l*-isoleucine, 9.0 gm. of crude carbobenzoxylglycyl-*l*-isoleucine were obtained. This was purified by recrystallization from a mixture of ethyl acetate and petroleum ether; m.p. 114–115°.

$C_{18}H_{22}O_5N_2$ .	Calculated.	C 59.6, H 6.9, N 8.7
322.4	Found.	" 59.6, " 6.8, " 8.7
$[\alpha]_D^{25} = +14.3^\circ$ (5% in ethyl alcohol)		

*Enzymatic Studies*

The crystalline carboxypeptidase was prepared and recrystallized three times according to the directions of Anson (17). The determination of the enzymatic hydrolysis was made by measuring the liberated carboxyl groups in the manner described earlier (5), except that the hydrolysis was carried out at 25° in all cases.

The authors wish to express their thanks to Dr. William H. Stein for samples of the *d* peptides used in this investigation and to Dr. Adalbert Elek for the microanalyses reported in this paper.

## SUMMARY

A study of the specificity of crystalline pancreatic carboxypeptidase has shown that several carbobenzoxyglycylamino acids differ greatly in their sensitivity to enzymatic action, depending on the nature of the terminal amino acid present in the substrate. This variation in sensitivity cannot be attributed to the presence, in the enzyme preparation, of several enzymes, since partial inactivation of carboxypeptidase results in a parallel decrease in enzymatic activity toward all the substrates studied.

The rate of action of carboxypeptidase on carbobenzoxyglycyl-*l*-phenylalanine is decreased by the presence of substances related in structure to this substrate.

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# THE EFFECT OF AMINO ACIDS ON THE MICROBIAL GROWTH INHIBITION PRODUCED BY THIENYLALANINE\*

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A recent paper from this laboratory (1) reported the inhibition of the growth of *Saccharomyces cerevisiae* by *dl*- $\beta$ -2-thienylalanine, an isostere of phenylalanine. This inhibitory action of thienylalanine was counteracted by phenylalanine. Thus, the thienylalanine was shown to act as an "antiphenylalanine" for this yeast. Tyrosine had no effect on the toxicity of the thienyl compound. Furthermore, a mixture of most of the other natural amino acids present in twice the concentration of phenylalanine required for reversal did not nullify inhibition of yeast growth by thienylalanine. This indicated that no other amino acid was so effective as phenylalanine in the prevention of the yeast growth inhibition due to thienylalanine but it did not exclude the possibility that, at much higher levels, other amino acids might counteract this toxicity. We wish to report here the effects of thienylalanine on *Streptococcus faecalis*, *Lactobacillus arabinosus* 17-5, *Escherichia coli*, and *Saccharomyces cerevisiae*, and the effects of high concentrations of most of the known naturally occurring amino acids on the inhibitory action of thienylalanine on the last two organisms.

## EXPERIMENTAL

*Inhibition of Growth of Saccharomyces cerevisiae*, Fleischmann's Strain 139—The technique followed in the yeast growth experiments was the same as that described previously (1). The medium employed in the work on yeast was a modification of that used by Snell, Eakin, and Williams (2). In the yeast growth experiments, 6.0 cc. of the medium were added to the various addenda dissolved in a volume of 1.0 cc., making a total volume of 7.0 cc. per assay tube (20  $\times$  150 mm.). The medium is shown in Table I.

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When this medium, containing the additional amino acids specified, was used, the amount of yeast growth measured turbidimetrically gave a reading of 240 units on the Klett-Summerson colorimeter. When the amino acids with the exception of aspartic acid were omitted from the medium, a reading of about 130 units was obtained. The additional amino acids did not appear to possess an effect, however, on the toxicity of thienylalanine.

TABLE I  
*Composition of Medium*

Compound	Amount	Compound	Amount
KH <sub>2</sub> PO <sub>4</sub> .....	20 gm.	l(+)-Aspartic acid.....	3.6 gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	2.5 "	l(-)-Asparagine.....	1.6 "
CaCl <sub>2</sub> ·2H <sub>2</sub> O.....	2.5 "	l(+)-Glutamic acid.....	1.6 "
H <sub>3</sub> BO <sub>3</sub> .....	10 mg.	l(+)-Lysine·HCl.....	1.6 "
ZnSO <sub>4</sub> ·7H <sub>2</sub> O.....	10 "	l(+)-Arginine·HCl.....	1.6 "
MnCl <sub>2</sub> .....	10 "	l(-)-Histidine·HCl·H <sub>2</sub> O...	1.6 "
TiCl <sub>3</sub> .....	10 "	l(-)-Proline.....	1.6 "
FeCl <sub>3</sub> .....	5 "	l(-)-Hydroxyproline.....	1.6 "
CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	1 "	Inositol.....	50 mg.
KI.....	1 "	Vitamin B <sub>6</sub> .....	0.2 "
Sucrose (purified).....	200 gm.	Biotin.....	16 γ
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	30 "	H <sub>2</sub> O to.....	10 liters
Glycine.....	1.6 "	Vitamin B <sub>1</sub> *.....	0.2 mg.
dl-Serine.....	1.6 "	dl-Calcium pantothenate*†	60 "
dl-Threonine.....	1.6 "		

\* Added after the medium is sterilized.

† When β-alanine was present in the medium instead of pantothenic acid, many of the amino acids inhibited the growth of the yeast. In the amount of 1.5 mg. per 7 cc. of medium, threonine, asparagine (3), aspartic acid, alanine, isoleucine, serine, or glycine gave pronounced inhibition. In the presence of calcium pantothenate, no inhibition by these amino acids was observed. A similar relationship between amino acids and β-alanine and its effect on the growth of a number of yeasts have been reported by Sarett and Cheldelin (4).

The effect of increasing amounts of dl-thienylalanine on the growth of yeast in the amino acid-supplemented medium is shown in Fig. 1, Curve 1. Also included are the inhibition curves in the presence of various levels of dl-phenylalanine. From these data, it can be seen that 42.5 γ of thienylalanine per 7 cc. reduced the growth to 50 per cent of normal. The ratio between the additional thienylalanine required and the amount of phenylalanine added remained constant over a wide range of phenylalanine concentrations, as is illustrated in Table II.

The yeast growth curves of Fig. 2 show the effect of increasing amounts of phenylalanine on the toxicity of various levels of thienylalanine. These

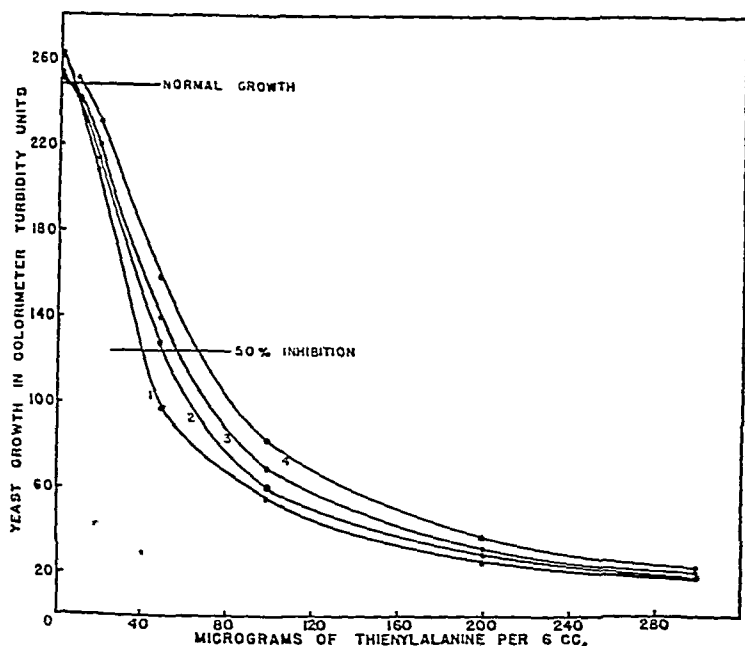


FIG. 1. Thienylalanine inhibition of yeast growth. Curve 1, with no *dl*-phenylalanine in the medium; Curves 2, 3, and 4, with 10  $\gamma$ , 20  $\gamma$ , and 30  $\gamma$ , respectively, of *dl*-phenylalanine per 7 cc.

TABLE II

*Relationship between Thienylalanine and Phenylalanine for Inhibition of Growth of Yeast*

<i>dl</i> -Phenylalanine added per 7.0 cc.	<i>dl</i> -Thienylalanine* added per 7.0 cc. for 50 per cent yeast growth inhibition	$\frac{\text{Thienylalanine}}{\text{Phenylalanine}}$
7	7	
10	8.5	0.85
20	16.3	0.82
30	24.5	0.82
mg.	mg.	
8	6.64	0.83

\* Amount of thienylalanine which had to be added above the amount required to produce 50 per cent inhibition without added phenylalanine.

curves indicate that the toxicity of fairly large amounts of thienylalanine can be counteracted by phenylalanine. The amount of phenylalanine required to counteract the toxicity of thienylalanine increased with the

amount of thienylalanine present and the ratio between these two quantities, as shown in Table III, remained quite constant.

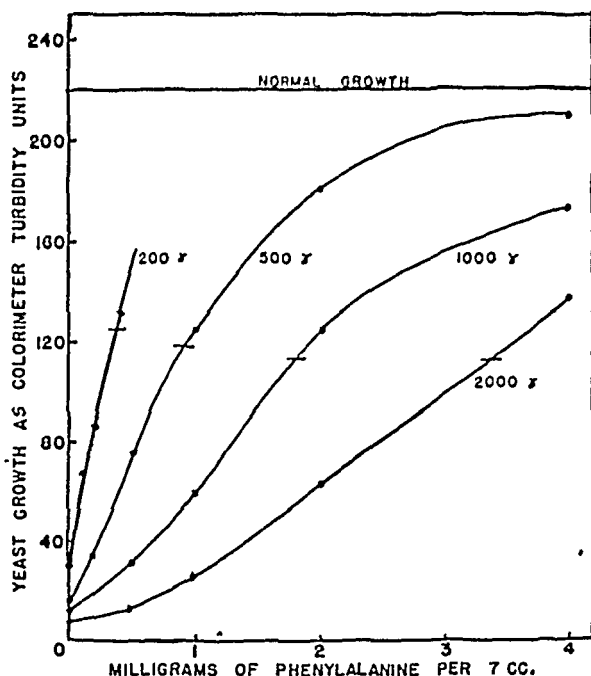


FIG. 2. Nullification of the toxicity of various *dl*-thienylalanine concentrations (indicated on each curve) by *dl*-phenylalanine. The short horizontal bars indicate the 50 per cent level of nullification of the thienylalanine toxicity.

TABLE III  
Nullification of Toxicity of Thienylalanine by Phenylalanine

<i>dl</i> -Thienylalanine per 7.0 cc.	<i>dl</i> -Phenylalanine required for 50 per cent nullification of toxicity	$\frac{\text{Thienylalanine}}{\text{Phenylalanine}}$
$\gamma$	$\gamma$	
200	370	0.54
500	920	0.54
1000	1800	0.55
2000	3300	0.61

*Inhibition of Growth of Escherichia coli*—The *E. coli*<sup>1</sup> used in these studies was grown on the synthetic medium described by Kalmanson and Bronfenbrenner (5) with the addition of the following amounts [of vitamins per liter: thiamine chloride 3 mg., pyridoxine hydrochloride 3 mg., calcium

<sup>1</sup> The *Escherichia coli* culture was obtained from Dr. James Neill of the Department of Bacteriology, Cornell University Medical College.

pantothenate 3 mg., nicotinic acid 6 mg. Best results were obtained if the medium was prepared daily. 5.0 cc. of medium were added to the various addenda dissolved in a volume of 1.0 cc. in the assay tubes (15 X 120 mm.). The tubes were plugged and autoclaved for 2 minutes at 15 pounds pressure and then inoculated aseptically with 1 drop of an *E. coli* suspension. The inoculum was kept constant by using suspensions of equal turbidity for each experiment. The *E. coli* suspension was prepared by centrifuging a 20 hour culture of *E. coli* in nutrient broth and adding it to enough sterile saline to give a suspension which was adjusted by the turbidity and contained 0.006 milliequivalent of nitrogen per cc.

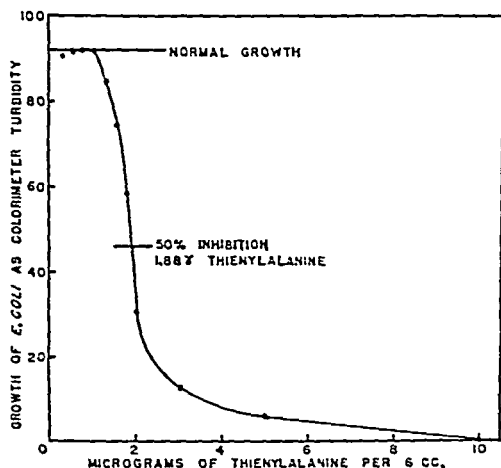


FIG. 3. The *Escherichia coli* growth inhibition curve of thienylalanine

(Kjeldahl). 1 drop of a 1:100 dilution of this suspension was added to each tube and the tubes were incubated for 16 hours at 37°. Growth was measured as turbidity in a Klett-Summerson photoelectric colorimeter.

The inhibition of the growth of *Escherichia coli* under these conditions is illustrated by the curve of Fig. 3. It will be noted that 1.88  $\gamma$  of thienylalanine inhibited the growth of *Escherichia coli* to 50 per cent of normal. When phenylalanine was added to the medium, more thienylalanine was required to maintain the growth level at 50 per cent of normal. The ratios of additional thienylalanine to phenylalanine, at levels of 50 per cent normal growth inhibition, increase with increasing amounts of phenylalanine. With levels above 5  $\gamma$  of phenylalanine per 6 cc., the ratio of thienylalanine to phenylalanine appears to be fairly constant, as is illustrated in Table IV. The amounts of phenylalanine required to bring the

inhibition due to various amounts of thienylalanine back to 50 per cent of normal growth are listed in Table V and plotted in Fig. 4. These results indicate that the ratio of thienylalanine to phenylalanine remains constant only at the higher levels of thienylalanine.

*Effect of Thienylalanine on Streptococcus faecalis* (American Type Culture Collection, No. 4081)—This organism did not grow in the medium described by McMahan and Snell (6) for *Lactobacillus casei* if phenylalanine was

TABLE IV  
Inhibition of Growth of *Escherichia coli* by Thienylalanine in Presence of Phenylalanine

dl-Phenylalanine per 6 cc.	dl-Thienylalanine* for 50 per cent inhibition of growth of <i>Escherichia coli</i> per 6 cc.	Thienylalanine Phenylalanine
$\gamma$	$\gamma$	
1.0	5.9	5.9
2.0	16	8
5.0	100	20
10.0	260	26
50	1200	24

\* Amount of thienylalanine which had to be added above the amount required to produce 50 per cent inhibition in the absence of added phenylalanine.

TABLE V  
Nullification by Phenylalanine of Thienylalanine Inhibition of Growth of *Escherichia coli*

dl-Thienylalanine per 6.0 cc.	dl-Phenylalanine required for 50 per cent nullification of toxicity	Thienylalanine Phenylalanine
$\gamma$	$\gamma$	
5	2.0	2.5
50	2.5	20
100	5.0	20
500	22.5	22
2000	100.0	20

omitted. In the presence of 30  $\gamma$  of dl-phenylalanine per 6 cc., good growth was obtained in 24 hours and this growth was completely inhibited by 50  $\gamma$  of dl-thienylalanine. An additional 50  $\gamma$  of dl-phenylalanine nullified this toxicity.

*Effect of Thienylalanine on Lactobacillus arabinosus 17-5*—This organism grew well in the medium of McMahan and Snell (6) without added phenylalanine. Thienylalanine inhibited the growth of this organism and again the inhibition was nullified by the addition of phenylalanine.

*Effect of Amino Acids Other Than Phenylalanine on Thienylalanine*



**Toxicity on Yeast and *Escherichia coli***—To study the effect of amino acids other than phenylalanine, a level of thienylalanine was chosen which was large enough to give a definite amount of inhibition of growth and yet was small enough so that a high ratio of amino acids to thienylalanine would obtain. For *Saccharomyces cerevisiae*, 50  $\gamma$  of thienylalanine per 7 cc. were chosen; the toxicity of this amount of thienylalanine was almost completely nullified by 100  $\gamma$  of phenylalanine.

A level of 5  $\gamma$  of thienylalanine per 6 cc. was selected to test the effects of the amino acids on the toxicity of thienylalanine for *Escherichia coli*.

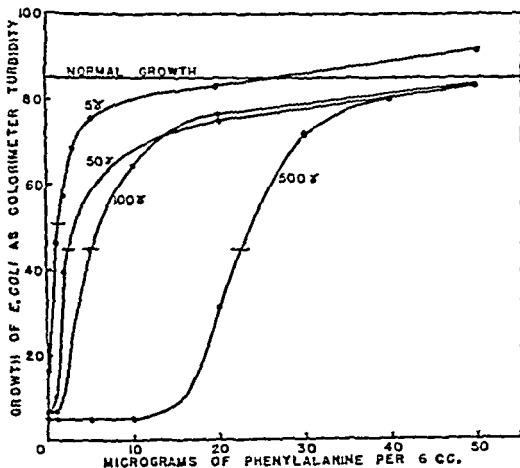


FIG. 4. Nullification of the thienylalanine inhibition of the growth of *Escherichia coli*. The short horizontal bars indicate the 50 per cent level of nullification of the thienylalanine toxicity.

This amount of thienylalanine almost completely inhibited the growth of *Escherichia coli* and was nullified by approximately 2  $\gamma$  of added phenylalanine.

The growth curves representing the counteraction of the thienylalanine toxicity for yeast by certain amino acids are plotted in Fig. 5. All of these amino acids were studied up to levels of 6.0 mg. per 7 cc. but are plotted only to levels of 2 mg. per 7 cc. The curves representing the counteraction of the thienylalanine inhibition of the growth of *Escherichia coli* by *dl*-phenylalanine, *L*-tyrosine, and *dl*-tryptophane are plotted in Fig. 6.

The effect of a series of amino acids on the toxicity of thienylalanine on both *Saccharomyces cerevisiae* and *Escherichia coli* is shown in Table VI.

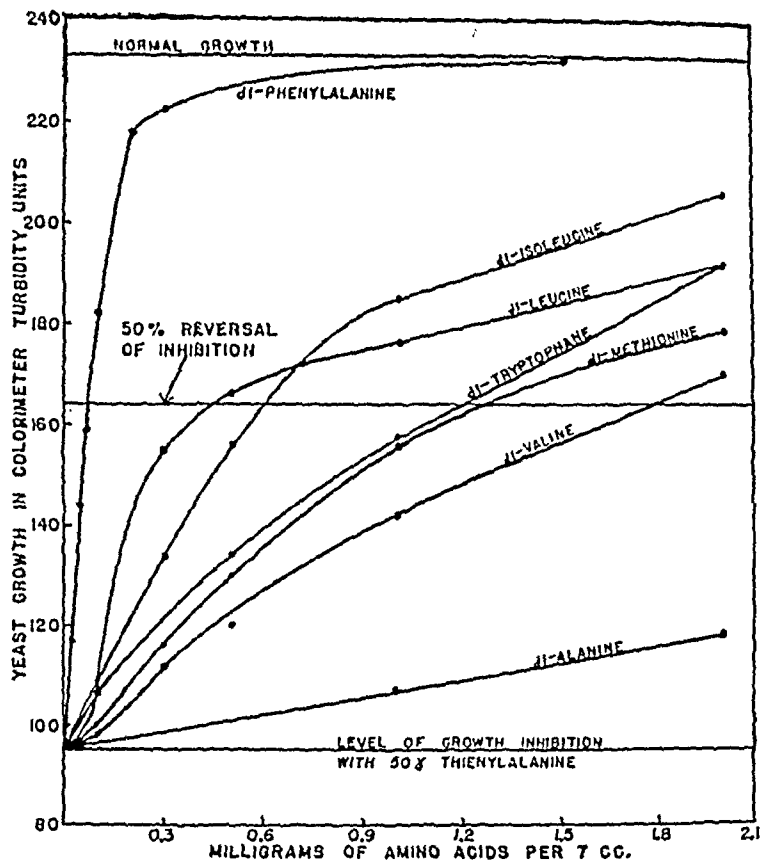


FIG. 5. The effect of various amino acids on the toxicity of 50  $\gamma$  of thienylalanine on the growth of *Saccharomyces cerevisiae*.

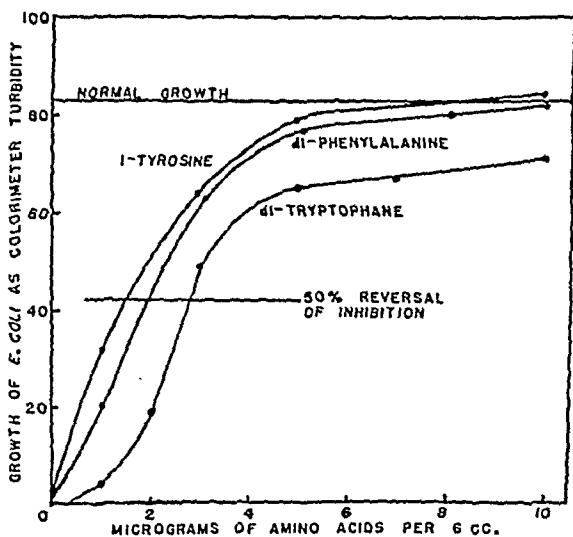


FIG. 6. The effect of *dl*-phenylalanine, *l*-tyrosine, and *dl*-tryptophane on the toxicity of 5  $\gamma$  of thienylalanine on the growth of *Escherichia coli*.

Those amino acids which counteracted the growth inhibition of either organism are listed first with their relative effectiveness compared with *dl*-phenylalanine taken as 100 per cent. The amino acids were compared with phenylalanine at the levels of amino acids which were necessary to obtain growth at a level halfway between normal growth and the growth in the presence of 50  $\gamma$  of thienylalanine in the case of yeast and of 5  $\gamma$

TABLE VI

Nullification of Thienylalanine Inhibition of Growth of *Saccharomyces cerevisiae* and *Escherichia coli* by Various Amino Acids

For <i>S. cerevisiae</i>			For <i>E. coli</i>		
Amino acid	Amount required for 50 per cent nullification of inhibition by 50 $\gamma$ thienylalanine	Effective-ness on molar basis	Amino acid	Amount required for 50 per cent nullification of inhibition by 5 $\gamma$ thienylalanine	Effective-ness on molar basis
	$\gamma$	per cent		$\gamma$	per cent
<i>dl</i> -Phenylalanine.....	75	100	<i>dl</i> -Phenylalanine...	1.9	100
<i>dl</i> -Leucine.....	450	13.2	<i>l</i> -Tyrosine.....	1.5	140
<i>dl</i> -Isoleucine.....	609	9.8	<i>dl</i> -Tryptophane*...	3.0	78
<i>dl</i> -Tryptophane*....	1160	8.0	<i>dl</i> -Leucine.....	980	0.15
<i>dl</i> -Methionine.....	1230	5.5	<i>l</i> -Histidine-HCl-		
<i>dl</i> -Valine.....	1740	3.1	H <sub>2</sub> O.....	700	0.25
<i>dl</i> -Alanine.....	6000	1.0	<i>dl</i> -Methionine.....	1550	0.11
Inactive: <i>l</i> -tyrosine, <i>l</i> -histidine			Inactive: <i>dl</i> -isoleucine, <i>dl</i> -valine, <i>dl</i> -alanine		

Inactive for both organisms: *dl*-glycine, *dl*-serine, *dl*-threonine, *l*-aspartic acid, *l*-glutamic acid, *l*-asparagine, *l*-cystine, *l*-lysine, *l*-arginine, *l*-proline, *l*-hydroxy-proline

\* *dl*-Tryptophane was obtained through the courtesy of The Dow Chemical Company.

with *Escherichia coli*. This is the growth level which represents 50 per cent nullification of the toxicity of thienylalanine.

It will be noted from the data of Table VI and Figs. 5 and 6 that, for yeast, no other amino acid was nearly as effective as phenylalanine, whereas for *Escherichia coli*, *l*-tyrosine and *dl*-tryptophane were also highly effective.

During this study, synthetic amino acids were used whenever possible, since several amino acids which were isolated from natural sources initially counteracted the thienylalanine inhibition of yeast growth, but did not demonstrate this effect when they were highly purified. Of the amino acids which were effective in counteracting the thienylalanine inhibition, only tyrosine and histidine were not synthetic products. The *l*-histidine

is only slightly active (0.25 per cent) for *Escherichia coli* as compared with phenylalanine, and the possibility of contamination with some of the more active amino acids is not ruled out. However, three different samples of *l*-histidine were found to be active.

In the case of phenylalanine, leucine, isoleucine, methionine, or tryptophane, the naturally occurring *l* isomer was twice as effective as the *dl* compound in preventing the inhibitory effect of thienylalanine on yeast growth. The same was true for phenylalanine, leucine, and tryptophane in the case of *Escherichia coli*. On the other hand, *dl*-methionine produced more of an effect on *E. coli* than half the amount of *l*-methionine. *d*-Methionine (by itself) only slightly counteracted the thienylalanine toxicity, but when both *l*-methionine and *d*-methionine were added together, the effect was approximately 60 per cent greater than would be expected from a simple additive effect. This would indicate that in the presence of *l*-methionine, the *d* isomer has considerable activity. Lampen, Jones, and Roepke (7) have reported that *d*-methionine will partially replace the requirement of *l*-methionine for the growth of a mutant of *E. coli* which requires methionine for growth.

#### SUMMARY

The "antiphenylalanine" properties of *dl*-thienylalanine have been studied with *Saccharomyces cerevisiae*, *Escherichia coli*, *Streptococcus faecalis*, and *Lactobacillus arabinosus*. For each microorganism the thienylalanine toxicity was counteracted by the addition of phenylalanine.

The effect of certain other amino acids on the thienylalanine inhibition of the growth of *Saccharomyces cerevisiae* and *Escherichia coli* has been investigated.

For *Saccharomyces cerevisiae*, none of the other amino acids were as effective as *dl*-phenylalanine in counteracting the toxicity of thienylalanine. *dl*-Leucine, *dl*-isoleucine, *dl*-tryptophane, *dl*-methionine, and *dl*-valine were effective but had to be present in levels 10 to 30 times as high as phenylalanine to produce comparable results.

For *Escherichia coli*, *dl*-phenylalanine and *dl*-tryptophane were almost equally effective in counteracting the toxicity of thienylalanine, and *l*-tyrosine was also effective. *dl*-Leucine, *l*-histidine, and *dl*-methionine were only slightly active.

All of the other naturally occurring amino acids which were tested were found to be without effect on either microorganism.

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# THE OXIDATION OF ASCORBIC ACID BY OXIDIZED ADRENALIN AND CYTOCHROME *c*\*

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Previous experimental results suggest that adrenalin and ascorbic acid are linked to a system of reductions and oxidations which play an important rôle in the formation of the aqueous humor (1). In order to obtain information about the enzymatic basis of these reactions we have investigated the distribution of some of the known oxidative enzymes in the tissue components of the ciliary body and of the chorioid plexus (2). It is possible, however, that the interaction of some of the presumptive mediators might be subject to an enzymatic catalysis that had so far escaped notice. In this connection we have examined the effect of various tissue preparations upon the reaction between ascorbic acid and oxidized adrenalin. We found that in the presence of the latter the disappearance of ascorbic acid was greatly accelerated and the oxygen uptake considerably enhanced by the addition of cytochrome *c*.

It has been generally accepted that cytochrome *c* accelerates the oxidation of various substrates by molecular oxygen only in systems which contain cytochrome oxidase. Our experiments suggested that an increase in the rate of oxidation of ascorbic acid by cytochrome *c* can also occur when oxidized adrenalin is added as the autoxidizable component instead of cytochrome oxidase. This possibility was studied in greater detail and the results are reported in this paper.

## EXPERIMENTAL

For the oxidation of adrenalin we prepared a solution which contained 1 mg. of a crystalline preparation (Parke, Davis) in 0.5 ml. of a 0.05 M phosphate buffer at pH 7.2. Between 2.0 and 3.5 ml. of this solution were pipetted into a Warburg flask and shaken at a rate of about 80 oscillations per minute. Oxygen uptake starts after a lag period of 1 to 2 hours, depending upon the purity and the age of the adrenalin preparation. At the same time the formation of red oxidation products can be observed. If not stated otherwise, we allowed the oxygen uptake to proceed until 80 to 110 c.mm. of oxygen had been consumed per mg. of adrenalin, which

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required 4 to 6 hours at a temperature of  $37.5^{\circ}$ . We assume that the solution contains after this time some unoxidized adrenalin and several oxidation products at different steps towards the maximum possible oxidation. As a matter of convenience and because of lack of better information, this mixture will be called oxidized adrenalin. Further comments concerning the oxidation products of adrenalin will be given in the discussion.

The rate of the oxidation of ascorbic acid was determined by measuring the oxygen uptake manometrically or by titrating the ascorbic acid with dichlorophenol indophenol. For the manometric determination the solutions of ascorbic acid or of the substances which were used in its place were pipetted into the side arms of Warburg flasks, while the other components of the system were pipetted into the main compartment. The content of the side arm was tipped into the main compartment after thermostabilization, and readings were taken every 5 minutes at  $37.5^{\circ}$  with air as the gas phase. It was found to be essential to shake the manometers at a high oscillation rate of about 120 per minute. All solutions were made up in 0.05 M phosphate buffer of pH 7.2, and the same buffer solution was used for the adjustment to 3.0 ml. of the fluid volume in the Warburg flasks. For all purposes we took double distilled water, with the second distillation carried out in an all-glass still.

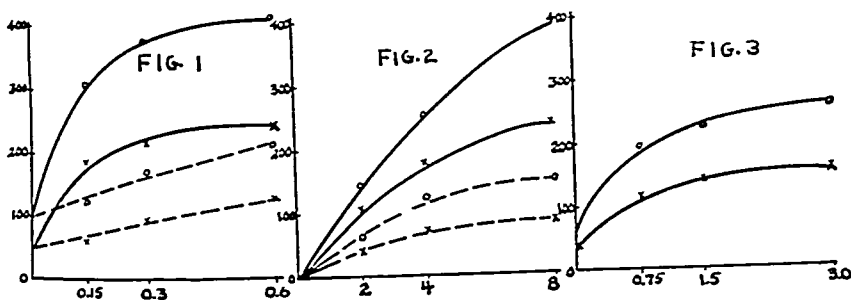
The titrations of ascorbic acid were carried out by transferring 0.1 or 0.2 ml. portions of the content of the Warburg vessels into 50 ml. beakers which contained 1.0 ml. of a 5 per cent solution of metaphosphoric acid. A slight opalescence developed when cytochrome *c* was present in the samples. With a little practice, we had no difficulty in recognizing the end-point of the titration in samples which showed this slight turbidity or the pale color due to the presence of cytochrome *c* or of oxidized adrenalin. The solution of dichlorophenol indophenol contained 72 mg. of the dye in 100 ml. of distilled water. In one series of experiments the rate of disappearance of ascorbic acid was determined only by titration. In this instance we used 50 ml. Erlenmeyer flasks instead of the Warburg flasks, since it is easier to remove aliquots from the solution while the reaction is in progress.

### *Results*

The acceleration of the oxidation of ascorbic acid by cytochrome *c* in the presence of oxidized adrenalin is demonstrated in Figs. 1 to 3. The rate of autooxidation of ascorbic acid is indicated in Figs. 1 and 3 by the values for the oxygen uptake at the intersection of the curves with the ordinate axis. It can be seen that oxidized adrenalin itself slightly accelerates the oxidation of ascorbic acid. Addition of cytochrome *c* enhances this acceleration up to 9 times, depending upon the concentration of the



components of the system. With ascorbic acid the increase in the rate of oxidation follows the increase in concentration up to the highest observed concentration ( $1.5 \times 10^{-2}$  M) and diminishes only slightly in the higher range (Fig. 2). In the case of cytochrome *c* and of oxidized adrenalin the acceleration reaches a more definite limiting value. The concentration of cytochrome *c* at this point is  $3.1 \times 10^{-5}$  M. Since it is not known which of the oxidation products of adrenalin are active in our system, a valid statement about the concentrations involved cannot be made. If one would assume that all the adrenalin has been transformed into active material, the concentration would correspond to  $1.1 \times 10^{-2}$  M. Probably it is much below this figure. The observed effect is greatly dependent



FIGS. 1 TO 3. Dependence of the oxygen uptake (c.mm. in 20 minutes, ordinate scale), in a system containing oxidized adrenalin, ascorbic acid, and cytochrome *c*, upon the concentration of the components. All figures refer to the content per flask; final fluid volume, 3.0 ml. of 0.05 M phosphate buffer, pH 7.2. Fig. 1, 6.0 mg. of ascorbic acid; 3.0 mg. of cytochrome *c*; abscissa, ml. of solution of oxidized adrenalin. Fig. 2, 0.2 ml. of solution of oxidized adrenalin; 3.0 mg. of cytochrome *c*; abscissa, mg. of ascorbic acid. Fig. 3, 0.2 ml. of solution of oxidized adrenalin; 3.0 mg. of ascorbic acid; abscissa, mg. of cytochrome *c*.

upon the pH. Our experiments were carried out at pH 7.0 to 7.5. At higher alkalinity measurements become less reliable because of the increasing autoxidation of ascorbic acid. When the acidity is increased, the effect is diminished and is practically absent at pH 5.5.

In order to exclude the possibility that an unknown amount of the oxidized adrenalin directly oxidizes the ascorbic acid, we placed oxidized adrenalin, corresponding to 1 mg. of adrenalin, and 3 mg. of cytochrome *c* in the main compartment of a Warburg vessel and 6 mg. of ascorbic acid in the side arm. The vessel was flushed for 10 minutes with nitrogen and after a further equilibration period of 5 minutes the ascorbic acid was tilted into the main compartment. After rapid shaking for 20 minutes ascorbic acid was determined in an aliquot by titration with dye. We could detect

no measurable disappearance of ascorbic acid. It follows that the presence of oxygen is essential for the reaction. The values obtained manometrically for the oxygen uptake after 20 minutes with different concentrations of oxidized adrenalin (Table I) were compared with the data for the disappearance of ascorbic acid as determined by titration with dichlorophenol indophenol. The titrations were carried out with aliquots of the same samples that were used for the manometric determinations. The dye titration measures only the first step in the oxidation of ascorbic acid, while the manometric values indicate the total oxygen consumption including the oxidations beyond dehydroascorbic acid. In the presence of cytochrome *c* the values obtained both manometrically and by dye titration decrease only by about 24 per cent (Column I) and 10 per cent (Column III) respectively with decreasing concentration of oxidized adrenalin.

TABLE I

*Comparison of the Effect of Cytochrome C upon Oxidation of Ascorbic Acid As Determined Manometrically and by Dye Titration*

Content per flask, cytochrome *c* 3 mg., ascorbic acid 6 mg.; final fluid volume, 3.0 ml. of 0.05 M phosphate buffer, pH 7.2; reaction time, 20 minutes.

Solution of oxidized adrenalin corresponding to adrenalin content of 2 mg. in 1.0 ml.	Oxygen uptake in mm $\times 10^{-2}$ ; manometric determination			Ascorbic acid disappearance in mm $\times 10^{-2}$ ; dye titration			(I) (III)	(II) (IV)
	Cytochrome c		$\frac{I}{II}$	Cytochrome c		$\frac{(III)}{(IV)}$		
	$\frac{+}{(I)}$	$\frac{-}{(II)}$		$\frac{+}{(III)}$	$\frac{-}{(IV)}$			
ml.								
0.6	1.82	0.94	1.94	2.17	1.15	1.89	0.84	0.82
0.3	1.64	0.74	2.22	2.16	1.18	1.83	0.76	0.63
0.15	1.38	0.41	3.37	1.95	0.86	2.26	0.71	0.48

Of the same order of magnitude (25 per cent) is the decrease in the absence of cytochrome *c* when the values are determined by dye titration (Column IV), while a much greater decrement (54 per cent) is observed in the manometric data (Column II), corresponding to an equally marked increase in the ratio of Column I to II. Calculation of the molecular ratios between oxygen consumption and ascorbic acid disappearance shows that at the highest concentration of oxidized adrenalin considerably more than 1 atom of oxygen is used per molecule of ascorbic acid. The oxygen uptake in excess of 0.5 M  $O_2$  per 1 M ascorbic acid is maintained at a high level by the added cytochrome *c* but decreases rapidly with decreasing concentrations of oxidized adrenalin in the absence of cytochrome *c* (Column II/IV). The results show that cytochrome *c* accelerates not only the first step in the oxidation of ascorbic acid but also the oxygen uptake of more than 1 atom of oxygen per molecule of ascorbic acid.

In order to obtain some information about the nature of the effect we tested several substitutes for each one of the three reacting substances.

*Cytochrome c*—In replacing cytochrome *c* we were interested to find out whether the effect could be produced with metals in the form of ions or bound in complex molecules of the type of cytochrome *c*. We added copper as cupric sulfate in a final concentration of from 0.25 to  $2.5 \times 10^{-4}$  M. The higher concentrations considerably accelerated the oxidation of ascorbic acid, but the acceleration was the same in the presence and absence of oxidized adrenalin. Using ferric chloride in the same concentrations we observed no acceleration of the autoxidation of ascorbic acid and the reaction was not catalyzed in the presence of oxidized adrenalin. Among the substances which are more closely related to cytochrome *c* we tested a crude preparation of hemoglobin. Oxalated human blood was washed in the centrifuge with physiological saline until the supernatant was free from oxalate and 1 ml. of the cell sediment was suspended in 10 ml. of distilled water. After completion of the hemolysis the ghost cells were centrifuged off and the supernatant was used as "hemoglobin" solution in these experiments. The concentration of the hemolysate (protein nitrogen) was adjusted to equal the concentration of the cytochrome *c* solution in our standard procedure. Under these conditions the reaction between oxidized adrenalin and ascorbic acid was not accelerated by the hemoglobin in the solution.

*Oxidized Adrenalin*—In a larger series of experiments we tried to obtain some information about the activity of adrenalin derivatives at different states of oxidation. The derivatives were produced by autoxidizing the adrenalin for different lengths of time in the usual way in Warburg flasks. The oxidation was followed *manometrically* and solutions were tested after the oxygen uptake had proceeded to the desired value. We obtained about half of the usual activity after an oxygen uptake of about 30 c.mm. per mg. of adrenalin and the full effect could be observed in most samples after an oxygen uptake of 50 c.mm. per mg. of adrenalin. These figures would correspond respectively to an oxidation of about 1 and 1.6 equivalents if one assumes that the oxidation progresses uniformly. The appearance of the red color, however, indicates that some of the adrenalin has undergone at least 4 equivalents of oxidation and, hence, that a part of the adrenalin must still be present in its original reduced state. Better clarification of this question will have to await the preparation of the various derivatives.

The compounds which were tested as substitutes for oxidized adrenalin were the oxidation products of hydroquinone, catechol, dihydroxyphenylalanine, and butanefrine<sup>1</sup> (terminal methyl group in adrenalin substituted

<sup>1</sup> Winthrop Chemical Company, Inc.

by butyl radical). These derivatives were obtained also by autoxidation in the same way as is described for adrenalin. Since the autoxidation of the first three substances at pH 7 proceeded too slowly to give the required oxygen uptake within a reasonable time, we used here pH 9.0, after having convinced ourselves that the oxidation products of adrenalin obtained at this pH have the same activity as those obtained at pH 7. We tested the solutions at two states of oxidation of the four compounds, corresponding to an oxygen uptake of 80 and 140 c.mm. per mg. of the respective substance. The oxidation products of hydroquinone, catechol, and dopa were completely ineffective. With dopa a precipitate formed in the presence of cytochrome *c* and it cannot be excluded that the failure in this case was due to the elimination of dopa from the solution. The oxidation product of butanefrine, on the other hand, was found to be only 20 per cent less active than oxidized adrenalin itself.

*Ascorbic Acid*—It was attempted to substitute for ascorbic acid other substances which are readily oxidized and, in turn, are active reducing agents, especially for the reduction of cytochrome *c*. We tested adrenalin itself as well as hydroquinone, catechol, and cysteine, substances which are frequently used as substrates in the cytochrome system. Cysteine was not only not oxidized but it even acted as an inhibitor for the catalyzed oxidation of ascorbic acid. With catechol, adrenalin, and hydroquinone no extra oxygen uptake was observed above the slow rate of autoxidation of these compounds. Isoascorbic acid is as effective as ascorbic acid but with glucose no  $O_2$  uptake was observed.

*Inhibitions of Cytochrome *c* Effect*—The effect was found to be sensitive to cyanide. In a concentration of  $3.5 \times 10^{-3}$  M the oxygen uptake was practically completely eliminated; with a concentration of about  $1.0 \times 10^{-3}$  M we obtained a decrease of about 50 per cent. The inhibitory action of cysteine has been mentioned in a previous paragraph. A complete inhibition was obtained at a concentration of  $5.0 \times 10^{-3}$  M and an inhibition of 20 per cent was obtained with  $7.5 \times 10^{-4}$  M. Two substances with pharmacological action synergistic to adrenalin, ephedrine and 2-methyltetrahydroisoquinoline hydrochloride (3), were also tested. 2 mg. of one or the other of these were added to the usual mixture in Warburg flasks. This amount was 5-fold of the amount of adrenalin present in these experiments, but no inhibitory or potentiating action could be detected.

We were interested in the effect of oxidized ascorbic acid on the cytochrome *c* effect. Ascorbic acid was oxidized by bubbling air through a solution of this substance at pH 8.0. The disappearance of reduced ascorbic acid was determined by dye titration. The sample was used when half of the ascorbic acid was present in oxidized form, the other

half in reduced form. To a control sample we added the same amount of reduced ascorbic acid without oxidized products. When tested in our system, the same acceleration of the disappearance of ascorbic acid by cytochrome *c* and oxidized adrenalin was observed in both samples. The presence of oxidized ascorbic acid has no inhibitory effect on this reaction.

#### DISCUSSION

The results of our experiments demonstrate that the addition of a cytochrome *c* preparation to a solution which contains oxidized adrenalin and ascorbic acid causes a considerable increase of the oxygen uptake and of the rate of disappearance of ascorbic acid. In discussing the nature of this effect the question arises as to the purity of our cytochrome *c* preparation. In consideration of the starting material from which the cytochrome *c* is obtained (hog hearts) it could be objected that the increased oxidation of ascorbic acid was caused by an incomplete elimination of cytochrome oxidase or by the presence of metal ions, especially of copper. In respect to the first possibility we should like to point out that the preparation of cytochrome *c* involves a precipitation by trichloroacetic acid and by acetone, which makes it very unlikely that any activity of the unstable cytochrome oxidase has remained. Moreover, no increase in the rate of oxidation of ascorbic acid could be discovered when a solution of cytochrome *c* was added to ascorbic acid in the absence of oxidized adrenalin. This latter result demonstrates more directly the absence of cytochrome oxidase in our preparations and differentiates the effect from the catalytic ascorbic acid autoxidation by various hematoporphyrins (4). It shows in addition that our cytochrome *c* preparation cannot contain active metal ions. We arrived at the same conclusion from results of experiments in which the cytochrome *c* was replaced by metal ions. Copper (as cupric sulfate) actually caused an increase in the rate of autoxidation of ascorbic acid. However, the increase was the same in the presence and in the absence of oxidized adrenalin. Ferric ions added in the same quantity as cupric ions did not cause a significant increase of the autoxidation, either in the presence or in the absence of oxidized adrenalin. Equally ineffective was a hemolysate which contains iron both bound to the porphyrin ring of hemoglobin and also as the usual ionic contamination.

Although the argument above makes it seem unlikely that the effect of our cytochrome *c* preparation is due to the presence of free metal ions or of cytochrome oxidase, our results with inhibitors would seem to indicate that a metal in some form is involved, since cyanide and cysteine, which are typical metal binders, act as strong inhibitors. Recent spectroscopic investigations suggest that cyanide can combine with cytochrome *c*, and therefore a participation of the porphyrin iron in cytochrome *c* has to be

considered (5). A possible rôle of  $H_2O_2$  which has been found to be formed in metal-catalyzed autoxidations of ascorbic acid cannot be excluded at the present time (6).

As an explanation of the accelerated oxidation of ascorbic acid one might be inclined to assume that the cytochrome *c* in our preparation is rapidly oxidized by one of the oxidative derivatives of adrenalin and is reduced by ascorbic acid. However, adrenochrome and other related compounds have a lower oxidation-reduction potential than cytochrome *c* itself, and these compounds could hardly act as efficient oxidizing agents for cytochrome *c*. Moreover, with cytochrome *c* as mediator it should be possible to replace ascorbic acid by any other substance which would reduce cytochrome *c*. Our results, however, indicate that hydroquinone, catechol, or adrenalin itself, very effective reducing agents for cytochrome *c* in the conventional cytochrome oxidase system, cannot act as substitutes for ascorbic acid in our experiments. This specificity of the reducing agent is striking, and it could mean that the reaction depends on the combination of oxidized adrenalin and ascorbic acid with a direct electron exchange between these compounds, the catalyst acting as an adsorbing surface which would bring the two substances into closer proximity and enhance thereby the probability of their interaction. Whatever the rôle of the catalyst may be, oxidized adrenalin seems to have a higher affinity than ascorbic acid, since the saturation concentration for the former is considerably lower than for the latter.

A connection between this effect and the physiological function of either adrenalin or ascorbic acid cannot be seen at the present time. In particular it should be pointed out that the compounds with adrenergic activity (ephedrine, isoquinoline) are without effect on the acceleration. On the other hand it should be kept in mind that similar mechanisms could play a rôle in the case of other physiological quinones, such as vitamin K.

#### SUMMARY

1. The rate of ascorbic acid oxidation in the presence of oxidized adrenalin is markedly enhanced by addition of cytochrome *c*.

2. The effect is specific in that cytochrome *c* cannot be replaced by copper or iron ions or by hemoglobin. Oxidized adrenalin cannot be replaced by oxidized hydroquinone or catechol and ascorbic acid cannot be replaced by such reducing agents as hydroquinone, catechol, cysteine, or glucose, while isoascorbic acid is equally effective.

3. The reaction is inhibited by cyanide and cysteine. Ephedrine, isoquinoline derivatives, and oxidized ascorbic acid have no inhibitory effect.

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## LETTERS TO THE EDITORS

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### THE LIPOTROPIC ACTION OF MANGANESE\*

Sirs:

In the course of studies to determine the effect of manganese and choline on bone formation in the rat, we found that manganese as well as choline prevents the deposition of excess fat in the liver. At a given level of choline, more fat was observed to be present in the livers of manganese-deficient rats than in the livers of rats receiving adequate manganese. The lipotropic action of manganese was much greater when the choline content of the diet was low, thus indicating an interaction between manganese and choline.

The basal diet used in the studies was similar to the low manganese diet described by Amdur, Norris, and Heuser.<sup>1</sup> The choline content was reduced by omitting the supplement of 0.2 mg. of choline per gm. of diet and reducing the whole milk powder content from 20 to 10 per cent. The carbohydrate, protein, and fat were increased to compensate for the lower level resulting from the reduced whole milk powder content. This gave a low choline, low manganese diet containing 0.1 mg. of choline and 0.3  $\gamma$  of manganese per gm. Choline was added to produce a medium choline (0.4 mg. per gm.), low manganese diet and a high choline (0.8 mg. per gm.), low manganese diet. A high manganese diet on each choline level was obtained by supplementing the corresponding low manganese diet with 0.25 per cent manganous chloride.

The fresh livers from each group were used as a composite sample. They were finely divided in a Waring blender, ground with anhydrous sodium sulfate, and extracted for 48 hours with chloroform. The fat was expressed as the percentage in fresh liver.

The data from five experiments are summarized in the accompanying graph. An analysis of variance showed that the reduction of liver fat by manganese was highly significant. As expected, choline likewise markedly

\* The authors wish to express their appreciation for the grant of the Snyder Fund and of a portion of the Sage Fund by the Graduate School of Cornell University in partial support of the research work presented in this report. They are also indebted to the Poultry Department of Cornell University for the use of their facilities in carrying out this investigation.

<sup>1</sup> Amdur, M. O., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, 69, 254 (1945).

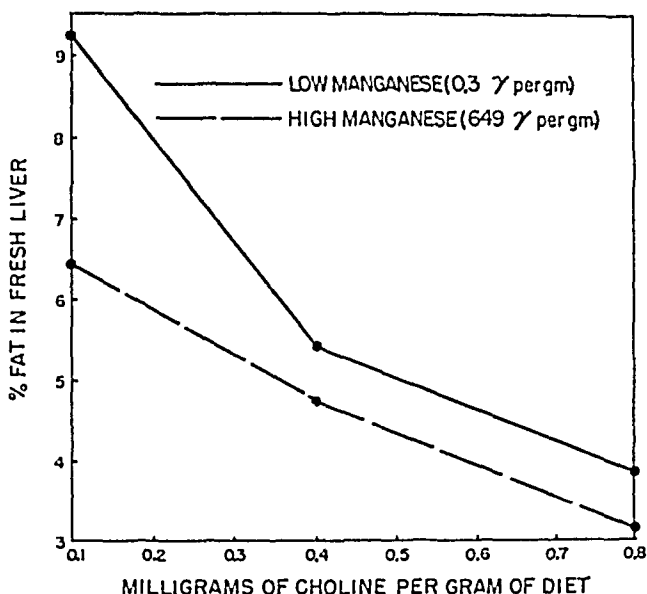


FIG. 1. The action of manganese and choline on liver fat. In all, 186 rats were used in these studies. Since there were three levels of choline and two levels of manganese used, each point on the graph represents a total of thirty-one animals. The pooled samples of four experiments contained the livers of six rats and that of the fifth experiment the livers of seven rats.

reduced liver fat. The manganese by choline interaction was found to be highly significant.

As further evidence that manganese has a lipotropic action, it was found that the presence of manganese in the diet caused a highly significant reduction in the percentage of fat occurring in the fresh bone. Choline also significantly reduced bone fat.

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# THE PARTIAL SYNTHESIS OF URINARY $\Delta^5$ -ANDROSTENE-3( $\beta$ ),-16,17-TRIOL

Sirs:

Recently Hirschmann<sup>1</sup> isolated from the urine of a patient with adrenocortical carcinoma a new triol of the androgen series. He brilliantly characterized this substance as a  $\Delta^5$ -androstene-3( $\beta$ ),16,17-triol. Marrian<sup>2</sup> subsequently reported that this triol is a constituent of normal human urine (both male and female). Marrian<sup>3</sup> had had this steroid in hand at a much earlier date but had been unable to establish an empirical formula for it.

In 1944 one of us,<sup>4</sup> using classical methods of organic chemistry, transformed estrone into the naturally occurring estriol (theelol). This transformation has been subjected to further study, and theelol may now be obtained from estrone in about 25 per cent over-all yield.

On turning to the androgen series and employing the set of reactions used to prepare theelol, it has been found that Hirschmann's triol, in satisfying yield, results as an end-product when dehydroisoandrosterone is substituted for estrone. This synthesis confirms for the triol the structure advanced by Hirschmann; the method of synthesis also leaves little doubt that the stereoisomeric configurations of the carbinols at C<sub>15</sub> and C<sub>17</sub> are identical in theelol and urinary androstenetriol. In fact, we are ready to state that the C<sub>15</sub>-OH and the C<sub>17</sub>-OH in theelol (and Hirschmann's triol) are in the trans geometric relationship, although this finding is contrary to the conclusion of Adam *et al.*<sup>5</sup> based upon surface film measurements. Theelol-3-methyl ether does not form a cyclic ketal with acetone and anhydrous hydrogen chloride, although such a derivative<sup>6,7</sup> is easily prepared from a steroid in which the C<sub>15</sub>-OH and the C<sub>17</sub>-OH are in cis relationship.

The chemical reactions employed to transform dehydroisoandrosterone into urinary androstenetriol are in general the same as those described<sup>8</sup> in the preparation of isoestriol-A; namely, nitrosation of 17-ketosteroid to the 16-oximino derivative, Stodola reduction of the 16-oximino com-

<sup>1</sup> Hirschmann, H., *J. Biol. Chem.*, **150**, 363 (1943).

<sup>2</sup> Marrian, G. F., *Nature*, **154**, 19 (1944).

<sup>3</sup> Marrian, G. F., and Butler, G. C., *Biochem. J.*, **38**, 322 (1944).

<sup>4</sup> Huffman, M. N., and Miller, W. R., *Science*, **100**, 312 (1944).

<sup>5</sup> Adam, N. K., Danielli, J. F., Haslewood, G. A. D., and Marrian, G. F., *Biochem. J.*, **26**, 1233 (1932).

<sup>6</sup> Butenandt, A., Schmidt-Thomé, J., and Weiss, T., *Ber. chem. Ges.*, **72**, 417 (1939).

<sup>7</sup> The present authors have had in hand a stereoisomeric estriol which very rapidly forms an acetonide under these conditions.

<sup>8</sup> Huffman, M. N., and Darby, H. H., *J. Am. Chem. Soc.*, **66**, 150 (1944).

pound to a mixture of isomeric  $\alpha$ -ketols, and further reduction of the appropriate  $\alpha$ -ketol to the 16,17-glycol. In this synthesis we obtained first the triacetate of androstenetriol (m.p. 184–185° uncorrected) and then saponified to the free triol (m.p. 257.5–260.5° uncorrected). Mixed melting point tests, kindly performed by Dr. Hirschmann, showed no depression of either synthetic compound when mixed with its counterpart of natural origin. Microanalysis<sup>9</sup> of synthetic  $\Delta^5$ -androstene-3( $\beta$ ),16,17-triol gave C 72.37, H 9.97 (calculated for  $C_{19}H_{30}O_3 \cdot \frac{1}{2}H_2O$ , C 72.34, H 9.91). Combustion of the synthetic triacetate showed C 69.41, H 8.46 (calculated, C 69.42, H 8.39).

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<sup>9</sup> Performed by Dr. E. W. D. Huffman, Denver.

## INEFFECTIVE UTILIZATION OF CONJUGATED PTEROYL-GLUTAMIC (FOLIC) ACID IN PERNICIOUS ANEMIA\*

Sirs:

The effectiveness of folic acid in pernicious anemia and the ability of normal subjects to subsist on ordinary sources of supply of folic acid suggest that in pernicious anemia some of the naturally occurring derivatives of the vitamin are not fully utilized.

Free vitamin was not released from the conjugated folic acid of yeast (pteroylheptaglutamic acid; kindly supplied by Dr. J. J. Piffner of Parke, Davis and Company) by incubation with normal human gastric juice at pH 7.0 or 4.5, and no reticulocyte response occurred in a patient with pernicious anemia in relapse who was given the heptaglutamate (1 mg.) orally each day for 10 days without, and for 11 days with, normal human gastric juice (100 cc. daily). However, daily administration of an equivalent amount (0.35 mg. orally) of pteroylglutamic acid caused a definite reticulocytosis (4.2 per cent).

In a similar patient a small reticulocyte response (3 per cent) followed the daily intramuscular injection for 10 days of 0.85 mg. of pteroylglutamic acid (average urinary elimination, 15 per cent), but no response was caused by daily injections of 2.5 mg. of the heptaglutamate for 12 days (average urinary elimination, only 1 per cent as free, none as the conjugate). One large intramuscular injection (30 mg.) of the conjugate caused no hematological response or urinary excretion during 12 days, while one injection of an equivalent amount of folic acid (11 mg.) caused prompt reticulocytosis (6.1 per cent) (average urinary elimination, 37 per cent).

A normal subject (average urinary elimination, 2.5  $\gamma$  of *L. casei* factor daily) given two daily intramuscular doses (2.8 mg.) of conjugate excreted 8.3 per cent as free *L. casei* factor (and none as the conjugate) in the urine, while of an equivalent amount of pteroylglutamic acid similarly injected, 16 per cent appeared in the urine.

Thus, in a normal individual, folic acid was released from pteroylheptaglutamate, while in patients with pernicious anemia the compound served inadequately as a source of folic acid and exerted no hematopoietic activity as such.

\* Discussed at the Symposium on Folic Acid, New York Academy of Sciences, May 29, 1946. This work was supported in part by a grant from the Lederle Laboratories, Inc., Pearl River, New York, who supplied the synthetic folic acid and the liver extracts.

Addition of purified liver extract<sup>1</sup> (0.007 cc.) to various dilutions of sternal bone marrow extracts from human patients with and without pernicious anemia has augmented frequently (50 to 150 per cent) the formation of *L. casei* factor from added heptaglutamate, *in vitro* at pH 4.5, but not at pH 7.0. These preliminary findings suggest that a constituent of liver extract may be a component of one of the conjugase systems or may counteract the effect of substances which inhibit the activity of the conjugase studied. Possibly this irregularly produced effect is attributable to the antipernicious anemia factor, but other functions of the factor are not excluded.

Such interpretations are consistent with the observation that the conjugate is ineffectively utilized in pernicious anemia and that injections of purified liver extract<sup>1</sup> have doubled the urinary excretion of *L. casei* factor by two patients with pernicious anemia. The data presented herein account, at least in part, for the failure of pernicious anemia individuals to derive adequate amounts of folic acid from the diet.

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<sup>1</sup> 15 unit liver extract; 1 cc. contains from 0.1 to 1  $\gamma$  of free *L. casei* factor and appears to be free of the conjugated vitamin.

# THE REPLACEMENT OF *p*-AMINOBENZOIC ACID IN THE GROWTH OF A MUTANT STRAIN OF *ESCHERICHIA COLI*

Sirs:

By x-irradiation of *Escherichia coli* we have obtained a mutant strain (No. 273-384) requiring *p*-aminobenzoic acid (PABA) for growth. The techniques for the production and isolation of mutant strains and the synthetic basal medium employed have been previously described.<sup>1</sup>

The response of the mutant to PABA is increased by an amino acid mixture (as used by Hac *et al.*<sup>2</sup>), of which methionine is the most important component. The addition of purines delays growth in the absence of the

*Response of Mutant Strain 273-384 to Pteroylglutamic Acid and to Thymine*

Compound		Supplement							
		None		Purines*		Amino acid mixture		Amino acid mixture + purines	
		16 hrs.	64 hrs.	16 hrs.	64 hrs.	16 hrs.	64 hrs.	16 hrs.	64 hrs.
<i>p</i> -Aminobenzoic acid	0.0005	15†	21	4	23	32	34	50	53
	0.01	56	74	63	76	69	74	81	78
Pteroylglutamic acid	0.1	0	1					6	13
	1.0	0	5					17	27
	10.0	9	14					73	76
Thymine	3.0	0	0	0	0	2	5	1	24
	30.0	1	5	0	0	6	10	44	45
	300.0	2	8	0	0	7	11	63	67

\* 5  $\gamma$  each of adenine, guanine, and xanthine per ml.

† Turbidity readings; 1 unit equals 13.4 million cells per ml.

amino acids but produces large turbidity increases in their presence.<sup>3</sup> The PABA requirement for half maximal growth at 16 hours falls from 0.0012  $\gamma$  per ml. on the basal medium to 0.00011  $\gamma$  per ml. in the presence of both supplements.

Pteroylglutamic acid, which contains the PABA nucleus,<sup>4</sup> has less than 0.001 per cent of the activity of PABA for this organism (see the table).

<sup>1</sup> Roepke, R. R., Libby, R. L., and Small, M. H., *J. Bact.*, **48**, 401 (1944)

<sup>2</sup> Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).

<sup>3</sup> Kohn, H. I., *Ann. New York Acad. Sc.*, **44**, 503 (1943)

<sup>4</sup> Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, **103**, 667 (1946).

This sample contained 0.2 per cent of free arylamine (as *p*-aminobenzoyl-glutamic acid); thus there is no proof that pteroylglutamic acid has any inherent growth activity for this strain.

Thymine is practically inactive alone or in the presence of purines, but in combination with the amino acids it can support light growth which can be subcultured. PABA can be effectively replaced by a combination of all three of these materials. The organism has been carried through ten transfers on this combination with 30  $\gamma$  of thymine per ml. With this supplementation both the mutant and the parent *E. coli* have proved highly resistant to sulfonamides. Good growth was observed after 24 hours in the presence of 1280  $\gamma$  per ml. of sulfadiazine.

The data indicate that at least with *E. coli* PABA functions in the synthesis of thymine, the purines, and one or more of the amino acids.

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# PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

## VIII. PREPARATION OF AN 11-KETO BILE ACID\*

Sirs:

In Paper VI of the present series<sup>1</sup> it was shown by exclusion of other possibilities that the "3,11-dihydroxy-12-ketocholanic acid" of Marker and Lawson<sup>2</sup> is actually 3( $\alpha$ ),12-dihydroxy-11-ketocholanic acid with a strong probability that the hydroxyl group at C<sub>12</sub> is in  $\beta$  configuration. We recently have been able to obtain additional evidence for the correctness of this conclusion. In addition the experiments reported here provide a new method for the preparation of an 11-keto steroid. Methyl 3( $\alpha$ )-methylsuccinoxy-11-keto-12( $\beta$ )-hydroxycholesterol<sup>3</sup> (m.p. 90–91°;  $[\alpha]_D^{25} = +63^\circ$  (CHCl<sub>3</sub>); C<sub>30</sub>H<sub>46</sub>O<sub>8</sub>, calculated, C 67.38, H 8.67; found, C 67.20, H 8.64) upon treatment with PBr<sub>3</sub> in chloroform solution was converted to methyl 3( $\alpha$ )-methylsuccinoxy-11-keto-12-bromocholanoate, m.p. 80–81°;  $[\alpha]_D^{25} = 0^\circ$  (CHCl<sub>3</sub>); C<sub>30</sub>H<sub>45</sub>O<sub>7</sub>Br, calculated, C 60.29, H 7.59, Br 13.37; found, C 59.61, H 7.69, Br 13.72. Reduction of the bromoketo ester with either zinc and acetic acid or with chromous chloride in acetone<sup>5</sup> yielded methyl 3( $\alpha$ )-methylsuccinoxy-11-ketocholanoate, m.p. 113–114°;  $[\alpha]_D^{25} = +63^\circ$  (CHCl<sub>3</sub>). Hydrolysis yielded 3( $\alpha$ )-hydroxy-11-ketocholanic acid, m.p. 218–221°,  $[\alpha]_D = +67^\circ$  (absolute ethanol), identical with the compound prepared by independent methods.

These results establish the position of the ketone group in the original compound at C<sub>11</sub>. Since the 11-keto-12-hydroxy acid can be readily prepared in good yield, the method is directly applicable for the formation of 11-keto steroids in a variety of compounds. We are at present engaged in further work upon this problem.

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<sup>1</sup> Gallagher, T. F., *J. Biol. Chem.*, **162**, 539 (1946).

<sup>2</sup> Marker, R. E., and Lawson, E. J., *J. Am. Chem. Soc.*, **60**, 1334 (1938).

<sup>3</sup> Wintersteiner, O., and Moore, M., *J. Biol. Chem.*, **162**, 725 (1946).

<sup>4</sup> All melting points are corrected.

<sup>5</sup> Julian, P. L., Cole, W., Magnani, A., and Meyer, E. W., *J. Am. Chem. Soc.*, **67**, 1728 (1945).



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